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THE EFFECT OF SALMINE ON BACTERIA[1]

THOMAS D. BROCK

Abstract

The bacteriostatic and bactericidal effects of salmine on various bacteria have been studied. Salmine has more bacteriostatic activity against Gram-positive than against Gram-negative bacteria. It is bactericidal in water but not in broth, and this bactericidal action occurs against both Gram-positive and Gram-negative bacteria. It has been shown that salmine causes agglutination of washed suspensions of certain bacteria and this agglutination is not correlated directly with the Gram stain. Salmine causes an increase in the turbidity of washed cells of all bacteria, Gram-positive and Gram-negative, and differs in this respect from the solutes sodium chloride and glucose, which affect only Gram-negative species.

A comparison has been made of the effects of salmine and polymyxin and it has been concluded that salmine may also act by attachment to the bacterial surface.

Introduction

The protamines are a group of small molecular weight proteins usually associated with nucleic acids in the cell. They are obtained in large quantities from ripe sperm cells, especially of fish. Salmine is the protamine from ripe salmon sperm. Its molecular weight has been reported variously as 2855 (Gortner and Gortner (7)) or 7000 (Velick and Udenfriend (16)). It consists of about 85% arginine with small amounts of valine, serine, and proline. It has no free amino groups, proline being at the beginning of the polypeptide chain. It is quite basic owing to the guanidino groups of arginine. The material of Velick and Udenfriend (16) had 25% nitrogen and 20.7% sulphate. The protamines are heat stable and can be autoclaved. They are water-soluble and form complexes or salts with many acidic substances, such as cephalin (Chargaff (2); Chargaff and Ziff (3)).

The bactericidal effects of salmine were studied briefly by Miller *et al.* (13) and by McClean (12). The combination of salmine with phosphorylase has been shown to result in the inhibition of the enzyme (Krebs (9); Madsen and Cori (10)).

The present work was undertaken in order to examine in more detail some of the effects of salmine on bacteria. Of particular interest was the possible differential effects of salmine on Gram-positive and Gram-negative bacteria. Also, it was felt that a comparison of the effects of salmine with those of other basic polypeptides, such as polymyxin, might be interesting.

[1]Manuscript received November 19, 1957.
Contribution from the Department of Biology, Western Reserve University, Cleveland 6, Ohio.

[The previous number of Can. J. Microbiol. (4, 1–64) was issued January 31, 1958.]

Methods

The following bacteria were used in these experiments: *Escherichia coli* ATCC 26, *Salmonella typhosa* ATCC 167, *Aerobacter aerogenes* ATCC 8308, *Proteus vulgaris* ATCC 8427, *Klebsiella pneumoniae* ATCC 10031, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella schottmuelleri* ATCC 9149, *Shigella dispar* Michigan State Department of Health No. 2214, *Staphylococcus aureus* Upjohn 76, *Staphylococcus aureus* Upjohn 606 (resistant to novobiocin, penicillin, oxytetracycline, chlortetracycline, tetracycline, and streptomycin), *Streptococcus hemolyticus* Upjohn 147, *Staphylococcus albus* ATCC 151, *Streptococcus fecalis* ATCC 6057, *Bacillus subtilus* 154A-GMS-3.

The bacteria were grown in 100 ml. of brain heart infusion broth (Difco) at 37° C. in 500 ml. flasks on a rotary shaker at 280 r.p.m. Cells were harvested by centrifuging and washed twice.

Turbidity was measured in a Beckman DU spectrophotometer at 500 mμ or with a Lumetron colorimeter.

The protamine used was salmine sulphate obtained from the Eli Lilly Company, lot 4491C.

Bacteriostatic effects were determined by growing the bacteria in brain heart infusion broth in tubes for 18 hours and recording the concentration of protamine which gave complete absence of visible growth.

Bactericidal effects were determined by making viable counts in brain heart infusion 1.5% agar with water as diluent. All bactericidal studies were carried out at 37° C.

Results

Antibacterial Properties of Salmine

The bacteriostatic effect of salmine for various organisms is shown in Table I. It is moderately bacteriostatic against most of the Gram-positive organisms, and weakly bacteriostatic against most of the Gram-negative organisms. Of interest is the lack of activity against *S. fecalis* and the fairly good activity against *P. aeruginosa*. A similar phenomenon is noted for these two organisms with polymyxin and neomycin. Salmine would not be considered an exceptionally active antibacterial agent on the basis of the data in Table I.

Salmine was rapidly bactericidal when the bacteria were suspended in water. In brain heart infusion broth it was not bactericidal (Table II). A similar phenomenon has been noticed by Gunnison *et al.* (8) for neomycin and polymyxin. Apparently broth constituents protect the cell against the bactericidal action.

Agglutination of Washed Cells

Washed cells of bacteria were reconstituted to twice the density obtained after overnight growth at 37° C. in brain heart infusion broth (Difco). Upon dilution 1/1 with the test substances, the original cell density was then obtained. It was observed that if cells were allowed to stand at room temperature for 15 minutes, agglutination occurred in the presence of salmine with certain bacteria. In Table III are the results of a series of experiments

TABLE I

BACTERIOSTATIC EFFECT OF SALMINE SULPHATE IN BRAIN HEART INFUSION BROTH AT 37° C.

Organism	Minimum inhibitory concn., μg./ml.
S. hemolyticus	64
S. fecalis	>1000
S. albus	8
S. aureus	125
S. aureus 606	64
B. subtilis	8
S. typhosa	125
S. marcescens	>1000
K. pneumoniae	>1000
A. aerogenes	500
S. schottmuelleri	1000
E. coli	>1000
P. aeruginosa	32
S. dispar	>1000
P. vulgaris	>1000

TABLE II

BACTERICIDAL EFFECTS OF 200 μG./ML. SALMINE SULPHATE AFTER 1 HOUR AT 37° C. IN WATER AND BROTH (DIFCO BRAIN HEART INFUSION)

Organism	0 hr.	Water control, 1 hr.	Salmine in water, 1 hr.	Salmine in broth, 1 hr.
E. coli	5×10^6	1×10^6	1×10^2	5×10^6
S. typhosa	1×10^5	1×10^5	0	1×10^5
K. pneumoniae	5×10^6	1×10^6	2×10^3	5×10^6
S. fecalis	1×10^6	1×10^6	50	1×10^6
S. aureus	2×10^5	2×10^5	2	2×10^5

TABLE III

AGGLUTINATION OF WASHED CELLS OF VARIOUS BACTERIA BY SALMINE SULPHATE AND ITS REVERSAL. AGGLUTINATION OF WASHED CELLS BY HORSE SERUM

Organism	Salmine, 200 μg./ml.	Salmine, 200 μg./ml. + brain heart 3.7%	Salmine, 200 μg./ml. + NaCl 3%	Horse serum 10%
S. albus	+	+	+	+
S. aureus	+	+	+	+
S. aureus 606	+	−	−	+
E. coli	−	−	−	+
S. typhosa	−	−	−	+
S. dispar	−	−	−	+
K. pneumoniae	+	+	+	−
P. vulgaris	+	−	−	+
P. aeruginosa	+	+	+	−

NOTE: (+) Agglutination.
(−) No agglutination.

with this substance. It can be seen that salmine agglutinates all of the Gram-positive bacteria and certain Gram-negative bacteria. The agglutination was reversed by sodium chloride and brain heart infusion (and also by peptone and yeast extract) in certain of the Gram-positive and Gram-negative bacteria but not in others. The antibiotic resistant *S. aureus* strain differed from the antibiotic sensitive strain in this respect. It should be noted that *K. pneumoniae* was agglutinated in the presence of brain heart, even though it is not inhibited under these conditions (Table I). *K. pneumoniae* growing in the presence of any concentration of salmine above 50 μg./ml. agglutinated as it grew. This is an indication that agglutination by itself is not a sufficient explanation of bacteriostatic or bactericidal effects.

It was determined that minimum concentrations of salmine for agglutination differed with different organisms. Certain values obtained are shown in Table IV. The minimum concentrations for agglutination of *K. pneumoniae* and *P. aeruginosa* were unchanged in the presence of 1.5% peptone or 3% sodium chloride, while with *P. vulgaris* this value rose to greater than 200 μg./ml. This gives proof that in those species in which agglutination occurs in the presence of sodium chloride or peptone, this agglutination is completely unaffected by the additions.

TABLE IV

MINIMUM CONCENTRATION OF SALMINE SULPHATE NECESSARY FOR AGGLUTINATION WITH VARIOUS ORGANISMS IN WATER

Organism	Minimum concn., μg./ml.
P. vulgaris	50
S. aureus	200
S. aureus 606	200
E. coli	>10,000
K. pneumoniae	25
P. aeruginosa	50

Salmine agglutination differs from the agglutination brought about by a substance such as horse serum. This latter agglutination, which is unaffected by the presence of broth constituents, also occurs with different organisms. It is a slower agglutination requiring 1.5 to 2 hours for completion. Table III shows the results obtained with horse serum.

The agglutination caused by salmine is unrelated to the Gram reaction of the organisms. If this agglutination is due to some surface property of the cells, it is probably different from the surface properties determining the Gram stain.

Effect of Protamine on Turbidity Changes

Recently Mager *et al.* (11) have shown that Gram-negative, but not Gram-positive bacteria, show a marked increase in turbidity when suspended in solutions of various solutes such as sodium chloride and glucose. This increase is presumably a volume change of the cells due to the movement of water out of the cells. These turbidity changes were only noticed in viable cells.

In the present study, it was confirmed that these characteristic turbidity changes only occurred in Gram-negative organisms when sodium chloride and glucose were used. However, the situation when salmine was used is quite different (Table V). Both Gram-positive and Gram-negative organisms showed marked turbidity changes at concentrations of solute much lower than those of glucose or sodium chloride. In the presence of brain heart broth, these turbidity changes did not occur, were diminished, or were reversed.

Time course studies with *E. coli* indicated that with salmine the maximum turbidity developed in about 30 minutes. It then fell off and gradually returned to its original level. With sodium chloride and glucose, maximum turbidity was reached much faster, usually within 10 minutes. Also, decline in turbidity was more rapid than with salmine. Although the concentration of salmine used was 1.4×10^{-4} *M* (assuming a molecular weight of 7000), a decrease in the concentration to 1×10^{-6} *M* resulted in approximately the same increase in turbidity. It can be concluded that within this range, the turbidity change is little affected by concentration.

TABLE V

TURBIDITY CHANGES IN WASHED CELL SUSPENSIONS IN THE PRESENCE OF VARIOUS SOLUTES*

Organism	Glucose, 0.1 *M*	NaCl, 0.1 *M*	Salmine,† 0.00014 *M*	Salmine, 0.00014 *M* in brain heart infusion
S. albus	0	1	39	10
S. aureus	0	0.6	72	−14
S. hemolyticus	−0.3	0	68	−13
S. fecalis	−0.3	−0.2	75	10
E. coli	10	32	75	12
S. typhosa	0	22	38	−16
S. dispar	5	35	29	−10
K. pneumoniae	21	59	89	−33
P. vulgaris	26	46	33	0
A. aerogenes	13	27	54	−13
S. schottmuelleri	5.7	32	73	−12

*Results expressed as increase or decrease in optical density as per cent of control.
†Assuming a molecular weight of 7000.

Discussion

These turbidity experiments were done at an initial optical density at 500 mμ of around 0.200. At this low cell density, no agglutination occurred. Since the change in turbidity is rapid, it is quite possible that it is due to a direct effect of salmine on the cell surface resulting in an increase in permeability of the cell to water. If water diffused out of the cell it would decrease in size and this would result in an increase in turbidity (14).

Although Mager *et al.* (11) and Avi-Dor *et al.* (1) implicate increased osmotic pressure external to the cell as the cause of turbidity changes, it seems hardly likely that this is the case with salmine, since the concentrations used are quite low. It seems more probably that salmine is having a direct effect on the cell surface, resulting in increased permeability to water.

Salmine is a basic polypeptide. Interaction of the basic polypeptide antibiotic polymyxin with the bacterial surface has been demonstrated by Few and Schulman (6) and Few (5). A similar action has been implied by Gunnison *et al.* (8) for the basic antibiotics neomycin and streptomycin. A comparison of the turbidity changes induced by the three substances salmine, polymyxin, and neomycin is given for selected organisms in Table VI. It is apparent that in most cases salmine and polymyxin behave similarly, raising the turbidity in both Gram-positive and Gram-negative organisms. Neomycin seems to have a reverse effect, causing a decrease in turbidity (lysis?). It seems likely that salmine and polymyxin have a similar mode of action.

TABLE VI

TURBIDITY CHANGES INDUCED BY CERTAIN SUBSTANCES ON WASHED CELL SUSPENSIONS*

Organism	Salmine, 100 μg./ml.	Polymyxin, 1000 μg./ml.	Neomycin, 1000 μg./ml.	NaCl, 0.1 *M*
E. coli	68	41	−2.7	14
K. pneumoniae	46	58	−21	20
P. vulgaris	16	−5.3	−15	35
S. fecalis	16	16	−13	2.3
S. aureus	66	26	−3.3	−2.7
S. albus	38	46	−10	−3.8

*Results expressed as increase or decrease in optical density as per cent of control.

If salmine acts on the surface of the bacterial cell, it may possibly be a useful substance in studying the nature of the cell surface. The fact that it induces agglutination in certain organisms, but not in others, may indicate a certain similarity in their surface properties. That this similarity is not correlated with Gram reaction is interesting and instructive. There have been several attempts to relate the Gram reaction to surface properties (Stacey (15), Dubos (4)) and, in certain respects, this may be the case. However, the surface is probably not a homogeneous structure, and may indicate different properties with different reagents.

The turbidity changes of washed cells is an interesting phenomenon and may also make a useful technique in the study of the interaction of substances with the bacterial cell. That this seems related to the Gram stain in certain cases (sodium chloride, glucose) and not in others (salmine, polymyxin), further indicates the complex nature of the problem. Experiments on turbidity changes are rapid and easily set up. There would seem to be little reason why this technique should not become widely used. In the study of the biology of the whole cell, such simple and precise procedures are needed.

The activity of salmine should be compared with that of polymyxin. Polymyxin is also more bactericidal in water than in broth (Gunnison *et al.* (8)) but has a much greater activity against Gram-negative organisms.

These experiments indicate that there is room for much experimentation on the action and interaction of cationic substances on bacterial cells.

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THE OXIDATION OF ETHANOL AND ACETATE IN SOILS[1]

I. L. Stevenson and H. Katznelson

Abstract

The oxidation of ethanol and acetate by the soil microflora has been studied manometrically in intact soil. Ethanol was oxidized to acetate without a preliminary lag period. The rate of oxidation of this substrate remained relatively constant for one soil but varied markedly with soils of different fertility levels. Acetate was also oxidized directly in soils but oxygen consumption stopped far below the theoretical level for complete oxidation. Uptake of oxygen by soil in the presence of acetate was greatly enhanced by the addition of 2,4-dinitrophenol suggesting that a significant amount of acetate may be assimilated oxidatively.

Introduction

Recent investigations in this laboratory have demonstrated that the soil microflora *in situ* actively metabolized various organic substrates (4). In most instances oxidation began with an initial lag period followed by increasing oxidation rates with time. Further studies showed that with casamino acids (Bacto vitamin-free, acid hydrolyzed) as a substrate the oxidative pattern appeared to be adaptive. When 2,4-dinitrophenol was added during the lag phase, oxidation was stopped; addition of this compound during the logarithmic phase failed to inhibit oxidation. That this represents an adaptive phenomenon was further suggested by the fact that there was no significant change in numbers of organisms during the period of increasing metabolic activity.

Studies have been continued in a search for substrates whose oxidative rates might provide some index of the biological activity of soils. It was felt that such substrates should be oxidized directly rather than adaptively. Ethanol and acetate were found to be such substrates and the work reported herein deals with their oxidation in soil.

Materials and Methods

Warburg Techniques

A 4 g. sample of the 0.5–2.0 mm. fraction of a soil was placed in the main chamber of a conventional Warburg vessel. The soil was brought to 60% water holding capacity by the addition of water or substrate. "Accordion-pleated" pieces of filter paper were inserted in the center well and 0.2 ml. of 20% potassium hydroxide added. The vessels were then attached to their respective manometers and placed in the water bath at 30° C. Manometers remained static during the experimental period. Unless otherwise stated results are presented as accumulated oxygen uptake or carbon dioxide evolution with the appropriate endogenous values subtracted.

Substrates and inhibitors were usually added directly to the soils so that the total volume of liquid added was equal to 60% water holding capacity.

[1]Manuscript received December 5, 1957.
Contribution No. 451 from the Bacteriology Division, Science Service, Canada Department of Agriculture, Ottawa.

Additional amounts of substrates and inhibitors (up to 0.5 ml.) could be added from the vessel side-arm at any time during the experimental period.

Manometric determinations of carbon dioxide evolution by the soils were carried out by the "direct method" (7).

Soils

The soils used in these studies were selected from various sites in Carleton County, Ontario. Uplands sand (Us), Manotick sandy loam (Ms), Kars gravelly sandy loam (K), and soil X (from the fertility plots of the Central Experimental Farm) are representative of the arable soils in this area. The North Gower soil (NG) is classified as a Brown Forest soil and was obtained from a deciduous wood lot.

Inasmuch as soils arriving from the field exhibited considerable variation in moisture content, they were air-dried for a short period before sieving and weighing. In our experience, soils brought rapidly to apparent dryness by a circulating fan (5–20 minutes) did not show the striking respiratory anomalies of completely air-dried soils (6).

Results

Ethanol Metabolism in Soil

When the oxidation of ethyl alcohol was first observed in soil, experiments were undertaken to determine the effect of substrate concentration. It was found that with concentrations up to 2%, a direct oxidation occurred with the

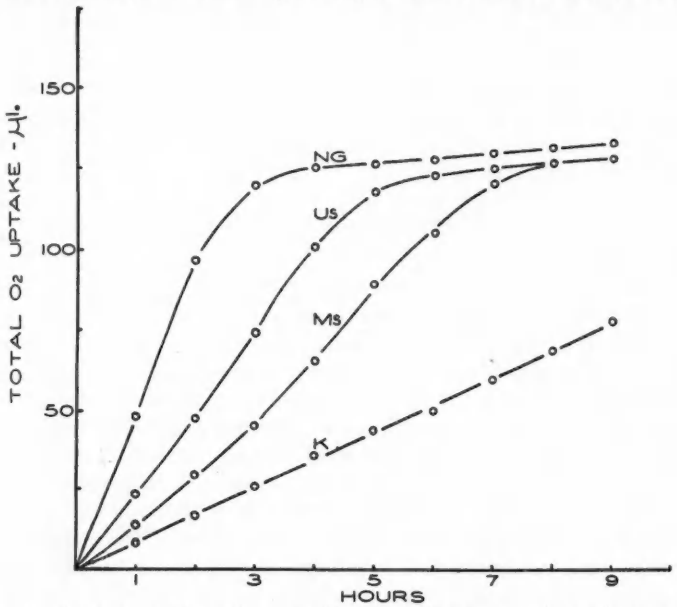

FIG. 1. Oxidation of ethanol in four soils. Each Warburg vessel contained 4 g. soil; 5 µM. ethanol in 1.5 ml. water for soils Us, Ms, and K and in 2.0 ml. water for soil NG; 0.2 ml. 20% KOH. Temperature 30° C.

rate of oxidation increasing as the concentration decreased. Working concentrations of 5–10 μM. per 4 g. of soil were finally selected and Fig. 1 illustrates the oxidation of 5 μM. ethanol in four soils. In each case the substrate is oxidized directly at an almost linear rate. In less active soils a slight increase in rate may occur after several hours but no evidence of a true lag period has been noted. Fig. 1 also illustrates that in the case of soils NG, Us, and Ms a break occurs in the oxidation curves followed by oxidation at a much reduced rate. This is again noted in Fig. 2, in which the decomposition of two concentrations of ethanol in two different soils is illustrated. Slightly lower initial rates are noted in both soils with 10 μM. of substrate; with this concentration a break in oxidation appears in both soil X and NG when oxygen uptake has reached a level of approx. 200–225 μl. With 5 μM. of ethanol

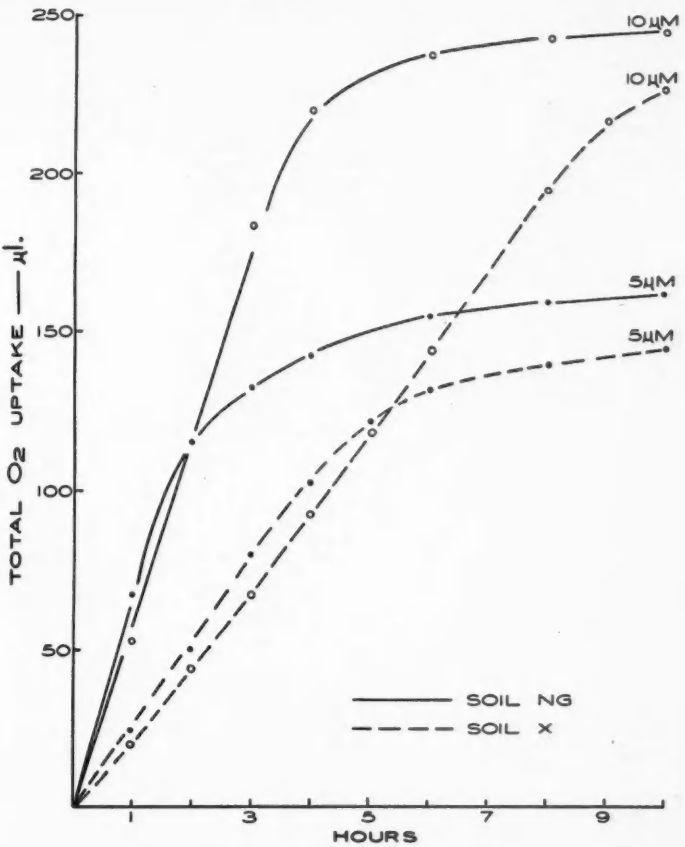

FIG. 2. Oxidation of 5 and 10 μM. ethanol in two soils. Each vessel contained 4 g. soil; 5 or 10 μM. ethanol in 1.5 ml. water for soil X and in 2.0 ml. water for soil NG; 0.2 ml. 20% KOH. Temperature 30° C.

this break appears at about one-half of this amount corresponding to an oxygen consumption of 1 mole per mole of substrate. From this data it would appear that ethanol is being oxidized to acetic acid according to the following equation:

$$C_2H_5OH + O_2 \longrightarrow CH_3COOH + H_2O \qquad [1]$$

Quastel and Scholefield have shown that in soils enriched with ethyl urethane, O_2 consumption with ethanol also corresponds to the amount required for oxidation to acetic acid (5).

Respiration studies involving the evolution of CO_2 and the uptake of O_2 with ethanol and sodium acetate as substrates are illustrated in Fig. 3. The oxygen uptake curve for ethanol shows the break appearing at the point suggested by the theoretical conversion of ethanol → acetate. With acetate as the substrate the ratio of O_2 uptake to CO_2 evolution is in keeping with the theoretical conversion of acetate → CO_2 and H_2O (1:1). No CO_2 is involved in the oxidation of ethanol to acetate and the observed CO_2 values in the presence of ethanol are likely due to a simultaneous oxidation of acetate as it is being formed. Extraction of ethanol-treated soils just subsequent to the break in oxidation resulted in the recovery of acetate.

The addition of 2,4-dinitrophenol (DNP) to the ethanol system resulted in a continued oxidation beyond the point where all the substrate had been converted to acetate.

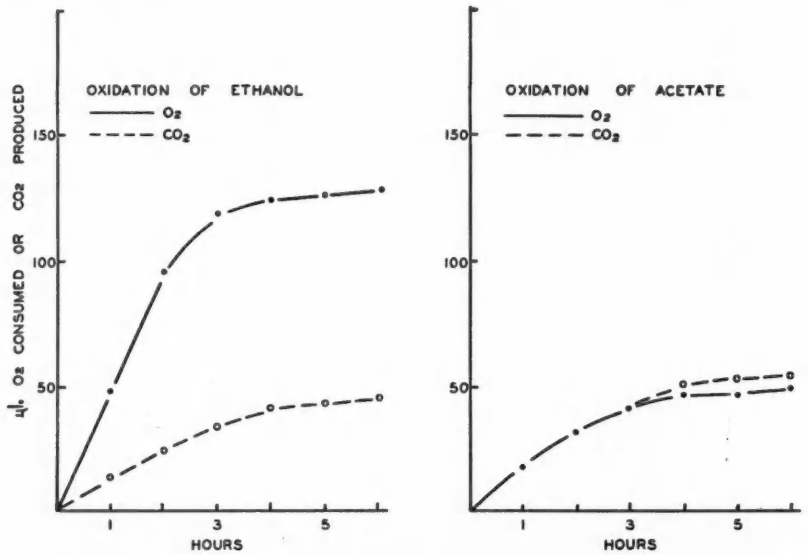

FIG. 3. Oxygen uptake and carbon dioxide evolution for soil NG supplemented with ethanol and acetate. Each vessel contained 4 g. soil; 5 μM. ethanol or sodium acetate in 2.0 ml. water. Temperature 30° C.

Acetate Metabolism in Soil

As with ethanol a direct oxidation of acetate occurs in soils. Fig. 4A illustrates the oxidation curves of 5 and 10 μM. of this substrate. Following an initial direct oxidation the rate of oxygen uptake falls off at a level much below the theoretical conversion to CO_2 and H_2O.

$$CH_3COOH + 2O_2 \longrightarrow 2CO_2 + 2H_2O \qquad [2]$$

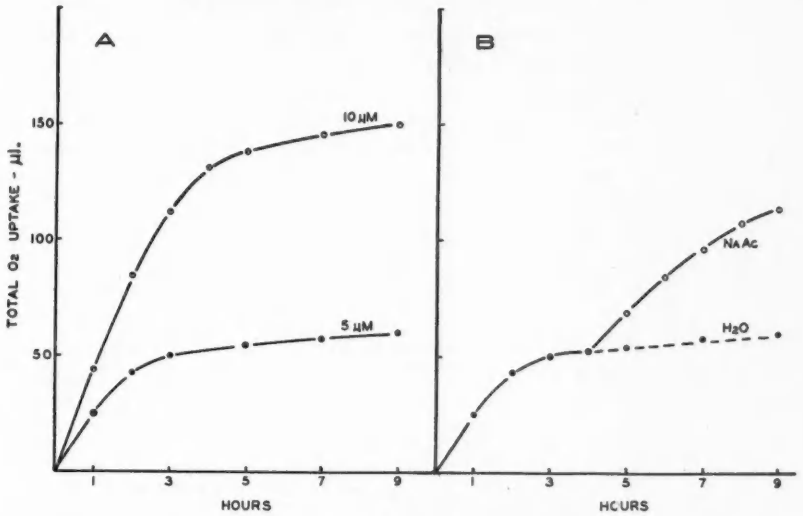

FIG. 4. (A) Oxidation of two concentrations of acetate in soil NG. Each vessel contained 4 g. soil; 5 or 10 μM. sodium acetate in 2.0 ml. water; 0.2 ml. 20% KOH. Temperature 30° C.
(B) Oxidation of acetate by soil NG. Vessels contained 4 g. soil; 5 μM. sodium acetate in water to 2.0 ml. Additional sodium acetate (5 μM. in 0.5 ml. water) tipped at 4 hours (solid line—open circle) with equal amount of water (0.5 ml.) tipped in control flasks (broken line—solid circle).

Despite the fact that oxidation has stopped short of completion the soil still retains the potential for further oxidation as illustrated in Fig. 4B. The addition of more acetate from the side-arm subsequent to the break results in renewed uptake of O_2 at approximately the original rate and to the same extent.

From these data it would appear that complete oxidation of acetate to CO_2 and H_2O does not occur. Working with *Pseudomonas calco-acetica* Clifton (3) has shown that acetate may be assimilated oxidatively and has demonstrated that in the presence of certain inhibitors which prevent this process a more complete oxidation of acetate takes place. Fig. 5 illustrates the effect of DNP on the oxidation of two concentrations of acetate in soil. With both concentrations of the substrate oxygen uptake levels are more than doubled before the oxidative rates decrease. Barker (1) and Clifton (3) have demonstrated

that the percentage assimilation from a given substrate varies with the nature of the organism. With acetate two possibilities exist for oxidative assimilation:

$$CH_3COOH + O_2 \longrightarrow CO_2 + H_2O + (CH_2O) \quad [3]$$

$$2CH_3COOH + 3O_2 \longrightarrow 3CO_2 + 3H_2O + (CH_2O) \quad [4]$$

In equation [3] one-half of the oxygen required for the complete combustion of acetate is consumed whereas in equation [4] three-quarters of the total oxygen is required. Oxygen uptake values obtained in our studies with acetate fall far below these theoretical levels. At the same time complete oxidation of acetate is not accomplished even in the presence of DNP.

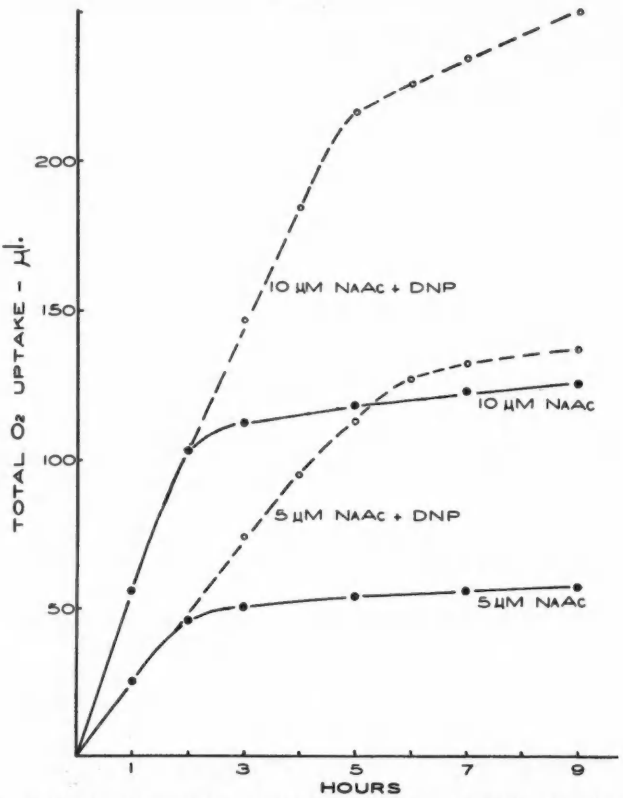

FIG. 5. Oxidation of acetate in the presence of 2,4-dinitrophenol. Vessels contained 4 g. of soil; 5 or 10 μM. of sodium acetate with and without 0.4 ml. $M/200$ 2,4-dinitrophenol; water to 2.0 ml.; 0.2 ml. 20% KOH. Temperature 30° C.

Discussion

The oxidation of ethanol in soil is interesting from several standpoints. Very few substrates have been found that are oxidized directly in soil without a preliminary lag or adaptive phase. Ethanol, however, appears to be directly

oxidized to acetate in soil. In this respect it is noteworthy that despite the heterogeneity of the soil population its accumulated activity results in a biochemical change very similar to that produced by pure cultures. Preliminary studies have shown that the oxidation of ethanol in soils parallels microbial activity as determined by the usual biological methods. For this reason the oxidation of this substrate has been selected as a potential index of microbiological activity. Trials have therefore been undertaken in which the decomposition of this substrate has been used to reflect changes in microbial activity during the decomposition of organic matter in soil.

Acetate metabolism in soil presents a much less straightforward picture. Although an initial direct oxidation occurs, the process stops at O_2 uptake levels far below the theoretical values for complete oxidation or for oxidative assimilation. The further oxidation of additional quantities of this substrate after the first oxidation has stopped precludes the possibility of enzyme inhibition or destruction, or cofactor limitation. In the presence of 2,4-dinitrophenol much higher oxygen uptake values are obtained but these are still below theoretical expectations. Nevertheless the effect of this inhibitor suggests that a large portion of acetate is being metabolized through oxidative assimilation.

A number of possibilities may be suggested to explain the loss of acetate to the oxidative system. It is possible that small quantities are irreversibly adsorbed in the soil. Secondly, some acetate may be converted non-oxidatively into other organic compounds. Acetate is an extremely active intermediate in numerous anaerobic reactions particularly those concerned with the formation of fatty acids (2). It may well be that a considerable amount of acetate is being diverted via such non-oxidative pathways.

Further studies into the fate of acetate in soil are being undertaken.

Acknowledgment

The authors gratefully acknowledge the effective technical assistance of Mrs. Kristine Orlik-Rückemann and Mr. Henry Malinowski.

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EFFECT OF AMINO ACIDS ON UTILIZATION OF SODIUM CASEINATE BY STREPTOCOCCUS LIQUEFACIENS AND STREPTOCOCCUS ZYMOGENES[1]

I. J. McDonald

Abstract

A strain of *Streptococcus zymogenes* and a strain of *Streptococcus liquefaciens* grew in a medium containing 10 amino acids and the latter organism was shown to produce an active proteinase in this medium. In the presence of glutamine and sodium caseinate, only five amino acids (glutamic acid, histidine, isoleucine, tryptophan, and valine) were required for growth. Under these conditions proteinase activity was barely demonstrable so that casein apparently was unable to induce proteinase synthesis to the same extent as were the five amino acids.

Introduction

Streptococcus liquefaciens and *Streptococcus zymogenes*, as shown previously (5), were unable to grow when sodium caseinate was the nitrogen source in an otherwise nutritionally complete medium, although they did use caseinate if small amounts of hydrolyzed casein were added to the medium. It was not known, however, whether the organisms required soluble nitrogen for initiation of growth prior to caseinate utilization or whether only a few amino acids were required to supplement those available from the unhydrolyzed protein. The present investigation was undertaken to determine which of the amino acids, essential for growth of these organisms, could be replaced with sodium caseinate and to determine the effect of such replacement on proteinase production by *S. liquefaciens*.

Materials and Methods

Cultures of *S. liquefaciens* ATCC No. 799 and *S. zymogenes* ATCC No. 6054 were maintained by monthly transfer in tryptone–tryptose agar (Medium II (4)).

The basal medium used in this study had the following composition per liter: glucose 4.0 g., sodium citrate trihydrate 20 g., sodium acetate trihydrate 20 g., monopotassium phosphate 1.0 g., dipotassium phosphate 1.0 g., modified Salt C (3) 20 ml., adenine sulphate 20 mg., guanine hydrochloride 20 mg., uracil 20 mg., xanthine 20 mg., thiamine hydrochloride 2.0 mg., riboflavin 4.0 mg., calcium pantothenate 4.0 mg., pyridoxine hydrochloride 4.0 mg., pyridoxamine dihydrochloride 2.0 mg., nicotinic acid 4.0 mg., *p*-aminobenzoic acid 2.0 mg., folic acid 0.04 μg., and biotin 0.04 μg. Five milliliters of basal medium was contained in 10 ml. of final medium. The final concentration of amino acids and peptides was equivalent to 0.1 mg. of the L-amino acid per ml. and that of sodium caseinate was 2.0 mg. per ml.

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Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa, Canada.
Issued as N.R.C. No. 4640.

Two per cent solutions of sodium caseinate (Nutritional Biochemical Corp.), purified and lyophilized as described previously (6), were boiled for 10 minutes, cooled, and adjusted to pH 4.6 with hydrochloric acid. The precipitate was washed several times with distilled water and redissolved with the aid of sodium hydroxide. The final solution (2%) at pH 6.8 was sterilized by Seitz filtration.

Five milliliter aliquots of basal medium plus amino acids were dispensed and the volumes adjusted so that after sterilization 0.5 ml. of Seitz-filtered glutamine (1.0 mg. per ml.) or 0.5 ml. of glutamine and 0.5 ml. of Seitz-filtered sodium caseinate could be added to give final volumes of 10 ml. per tube.

Cells for inocula were centrifuged from 18- to 24-hour cultures in tryptone–tryptose broth, washed three times, and diluted to a turbidity of 70% light transmittance with sterile, 0.9% saline. The standardized suspensions were diluted 1:100 with sterile saline and 0.1 ml. used to inoculate 10 ml. of medium. After incubation at 30° C., growth in media with amino acids was measured by determining the turbidity at 660 mμ, using a Coleman Junior spectrophotometer, and in media with sodium caseinate, by titrating the acid produced using 0.1 *N* sodium hydroxide and phenolphthalein indicator.

Media for proteolytic enzyme production of *S. liquefaciens* contained basal medium and various combinations of amino acids and sodium caseinate. Cells from 100 ml. of tryptone–tryptose broth were washed twice, resuspended in 2.5 ml. of sterile saline, and used to inoculate 100 ml. of sterile medium. Aliquots, taken immediately and after incubation for 6 hours at 35° C., were cooled and centrifuged at 0° C. The supernatant liquids were assayed for proteinase activity by measuring the tyrosine liberated from casein by the following procedure. The reaction mixture consisting of 5.0 ml. of casein substrate,* 5.0 ml. of culture supernatant, and 0.1 ml. of toluene was incubated at 35° C. Samples (3.0 ml.) were taken at 0 and 6 hours and added with shaking to 3.0 ml. of 0.6 *N* trichloroacetic acid. An aliquot of the trichloroacetic acid filtrate was added to 5.0 ml. of sodium carbonate solution (2), the volume was adjusted to 7.0 ml. with distilled water, and 3.0 ml. of phenol reagent (1) (diluted 1:3) was added. After incubation at room temperature for 10 minutes, the optical density was measured at 660 mμ and compared with that of a series of standard tyrosine solutions. One unit of enzyme has been defined arbitrarily as the amount of enzyme that liberates 1.0 μg. of tyrosine from casein in 6 hours at 35° C.

Results

Growth of *S. liquefaciens* and *S. zymogenes* was determined in a medium containing 19 amino acids and the effect of eliminating one amino acid at a time was observed. Under these conditions arginine, histidine, isoleucine, leucine, methionine, tryptophan, and valine were essential for growth while

*Casein prepared according to directions from Dr. F. H. Grutter: 3.0 g. of vitamin-free casein and 100 ml. of 0.05 *N* sodium hydroxide were steamed for 10 minutes and cooled, 30 ml. of 0.5 *N* monopotassium phosphate, 7.5 ml. of aqueous merthiolate (1:1000), and 140 ml. of water were added. The pH was adjusted to 7.4 and the volume to 300 ml.

threonine and serine stimulated early growth of both organisms. Alanine and glutamic acid were essential for *S. liquefaciens* but not for *S. zymogenes*. In a medium containing only the above 11 amino acids, both organisms grew (Table I) but under these conditions, alanine was no longer essential and could be omitted without affecting growth. When either serine or threonine was omitted, limited growth occurred but when any of the remaining eight amino acids was omitted, the organisms failed to grow. Therefore, the organisms grew in a medium containing 10 amino acids none of which could be omitted without markedly limiting the amount of growth.

TABLE I

GROWTH OF *S. liquefaciens* AND *S. zymogenes* WITH MINIMAL NUMBER OF AMINO ACIDS

(48 hours at 30° C.)

Amino acid omitted*	% incident light transmitted†	
	S. liquefaciens	*S. zymogenes*
None	52	56
DL-Alanine	58	55
L-Arginine HCl	99	99
L-Glutamic acid	100	100
L-Histidine HCl	98	99
DL-Isoleucine	100	100
L-Leucine	96	95
DL-Methionine	94	95
DL-Serine	78	84
DL-Threonine	89	81
DL-Tryptophan	99	99
DL-Valine	100	100

*Complete mixture contained the 11 amino acids listed.
†Representative data.

Previous results (5) showed that *S. liquefaciens* and *S. zymogenes* did not grow in a medium containing sodium caseinate and glutamine unless a source of amino acids was added. To determine which of the 10 essential amino acids could be supplied by sodium caseinate, a medium with 19 amino acids was prepared. By eliminating one amino acid at a time, the effect of substituting sodium caseinate was determined. The results (Table II) indicated that under these conditions caseinate replaced five amino acids and failed to replace three (histidine, isoleucine, and tryptophan). The results with arginine and valine were variable.

After some preliminary trials, it was found that *S. liquefaciens* and *S. zymogenes* grew in a medium with sodium caseinate, glutamine, glutamic acid, histidine, isoleucine, tryptophan, and valine (Table III). When either glutamine or glutamic acid was omitted, growth did not occur but it is not known why both were required under these conditions. When any of the remaining four amino acids was omitted, growth was very erratic. Thus sodium caseinate supported growth when it was supplemented with five amino acids and glutamine. To determine if these five amino acids were present as "free"

amino acids, sodium caseinate was adjusted to pH 4.5 and filtered. Neutralized filtrate equivalent to as much as 10 mg. of sodium caseinate per ml. did not support growth of *S. liquefaciens* or *S. zymogenes* in a medium containing glutamine, glutamic acid, histidine, isoleucine, tryptophan, and valine.

TABLE II

EFFECT OF SUBSTITUTION OF SODIUM CASEINATE FOR AMINO ACIDS ON GROWTH OF *S. liquefaciens* AND *S. zymogenes*

(90 hours at 30° C.)

	Ml. of 0.1 *N* NaOH/10 ml. of culture†	
Amino acid omitted*	*S. liquefaciens*	*S. zymogenes*
L-Glutamic acid	1.3–1.5	1.3–1.5
L-Leucine	1.3–1.5	1.3–1.5
DL-Methionine	1.0–1.1	1.1–1.2
DL-Serine	1.6–1.7	1.6–1.7
DL-Threonine	1.2–1.3	1.2–1.3
L-Arginine HCl	0.5–2.0	0.2–1.5
L-Histidine HCl	0.1–0.5	0.3–0.6
DL-Isoleucine	0.0–0.1	0.0–0.5
L-Tryptophan	0.2–0.6	0.2–0.6
DL-Valine	0.0–0.1	0.0–2.0

*Medium contained the 10 amino acids listed plus alanine, asparagine, aspartic acid, cysteine hydrochloride, glycine, glutamine, lysine hydrochloride, phenylalanine, proline, and tyrosine (DL, 0.2 mg./ml. and L, 0.1 mg./ml.) and sodium caseinate (2.0 mg./ml.).

†Range of values in two replicate experiments.

TABLE III

GROWTH OF *S. liquefaciens* AND *S. zymogenes* WITH SODIUM CASEINATE IN THE PRESENCE OF A LIMITED NUMBER OF AMINO ACIDS

(70 hours at 30° C.)

	Ml. of 0.1 *N* NaOH/10 ml. of culture†	
Amino acid omitted*	*S. liquefaciens*	*S. zymogenes*
None	1.9	1.9
Arginine, leucine, methionine, serine, and threonine	1.9	1.9
Five above and glutamic acid	0.0	0.0

*Complete mixture: arginine, glutamic acid, glutamine, histidine, isoleucine, leucine, methionine, serine, threonine, tryptophan, and valine plus sodium caseinate (2.0 mg./ml.).

†Representative data.

Furthermore, other attempts to demonstrate free amino acids in the unhydrolyzed sodium caseinate were not successful. Of the five amino acids supplied by caseinate, free arginine, leucine, and methionine were not detected by microbiological assay using *Leuconostoc mesenteroides* P-60. Materials for the determination of serine and threonine were not available but lysine, phenylalanine, and tyrosine were not detected with *L. mesenteroides* and tryptophan was not detected with *Lactobacillus arabinosus* 17/5, thus supplying additional evidence that unhydrolyzed sodium caseinate was devoid of free amino acids.

The ability of the organisms to utilize peptides was determined by substituting histidyl-histidine, DL-leucyl-glycine, DL-alanyl-DL-methionine, glycyl-DL-serine, glycyl-DL-threonine, glycyl-L-tryptophan, and glycyl-DL-valine for histidine, leucine, methionine, serine, threonine, tryptophan, and valine in a medium containing the other three essential amino acids plus alanine and glycine. *S. liquefaciens* grew more slowly with the peptides than with the amino acids but the final amount of growth was the same in both media. *S. zymogenes* grew at the same rate and to the same extent with peptides as with amino acids. In media with caseinate, glutamine, glutamic acid, and isoleucine, the peptides of histidine, tryptophan, and valine allowed as good growth of the organisms as did the free amino acids.

Proteinase production by *S. liquefaciens* was dependent upon the composition of the medium (Table IV). In the presence of 10 essential amino acids the organism produced 126 units of proteinase per ml. Although good growth occurred in a medium with five amino acids replaced by sodium caseinate, only 7.0 units of proteinase activity could be detected. Media that contained only five amino acids did not support growth and contained only 2.0 units of activity per ml. In another experiment, 52 units of enzyme activity were produced in a medium with 10 amino acids and 84 units of enzyme were produced when the medium contained 10 amino acids plus sodium caseinate. The presence of casein, therefore, did not interfere with the detection of enzyme activity.

TABLE IV

PROTEINASE PRODUCTION BY *S. liquefaciens*

Medium*	Units of proteinase per ml.† of culture filtrate
I 10 amino acids	126
II 5 amino acids + sodium caseinate	7.0
III 5 amino acids	2.0

*Basal medium (see text) containing 0.1 mg. of glutamine/ml.
I = 5 amino acids in III plus arginine, leucine, methionine, serine, and threonine (each 0.2 mg./ml.).
II = 5 amino acids in III plus 2.0 mg. of sodium caseinate/ml.
III = glutamic acid, histidine, isoleucine, tryptophan, and valine (each 0.2 mg./ml.).
†Representative data.

Proteinase, produced by *S. liquefaciens* in a complete amino acid medium, was concentrated from the cell-free medium by ammonium sulphate precipitation and dialysis. Sodium caseinate treated with this enzyme for 1 hour at 35° C. supported growth of *S. liquefaciens* and *S. zymogenes* whereas sodium caseinate treated with a boiled sample of this enzyme did not. Thus *S. liquefaciens* proteinase hydrolyzed casein sufficiently that the 10 essential amino acids became available to the organisms.

Discussion

Under the conditions employed, *S. liquefaciens* and *S. zymogenes* grew in a medium containing arginine, glutamic acid, histidine, isoleucine, leucine, methionine, serine, threonine, tryptophan, and valine. Omission of any one of these resulted in marked reduction or complete absence of growth and these 10 amino acids, therefore, were essential. These results differ somewhat from those of Niven and Sherman (7), who reported that alanine, lysine, and tyrosine were also essential. However, these authors observed that amino acid requirements of enterococci may vary among individual strains and this probably accounts for the differences noted.

The results indicated that although *S. liquefaciens* and *S. zymogenes* were unable to utilize sodium caseinate as a sole source of their essential amino acids, not all the essential amino acids had to be supplied to allow growth in the presence of the protein. Thus, as long as five of the 10 essential amino acids were present, the organisms obtained the remaining five (arginine, leucine, methionine, serine, and threonine) from caseinate. These amino acids then were made available to the organisms by hydrolysis of the protein. Since the proteinase from *S. liquefaciens* grown in a complete amino acid medium hydrolyzed casein sufficiently to support growth, the enzyme was capable of making the 10 essential amino acids available to the organism. However, in media with a limited number of amino acids, certain ones had to be present before the organism could grow with caseinate. Under these limiting conditions it is possible, firstly, that the organisms produced, from their metabolic pool of amino acids, enzyme that was sufficiently active to hydrolyze casein to yield some but not all the essential amino acids. This is supported somewhat by the behavior of the organisms towards glutamic acid which was obtained from casein when the medium was rich in amino acids but not when a few amino acids were present. Under these conditions, amino acids liberated would depend, not only upon the activity of the enzyme but also upon the specificity of the enzyme and the position of the amino acids in the protein molecule. Secondly it is possible that the organisms required some essential amino acids to enable them to initiate proteinase synthesis. This possibility is supported somewhat by the observation that the organisms did not use caseinate when a complete mixture of amino acids was altered by omitting either histidine, isoleucine, tryptophan, or valine. The peculiar behavior of the organisms towards arginine does not appear to be explained by either of the two possibilities above. It seems likely that it may be an effect of amino acid imbalance or antagonism.

S. liquefaciens was capable of producing proteinase in the absence of protein, an observation in agreement with those of Rabin and Zimmerman (8). However, the organism was incapable of producing maximum amounts of enzyme when caseinate replaced some of the essential amino acids. Proteinase production by *S. liquefaciens*, therefore, required amino acids (or peptides) and protein was unable to induce enzyme production to the same extent as were free amino acids.

Acknowledgment

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PROVISIONAL SHIGELLA SEROTYPES[1]

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Abstract

The results of continued biochemical and serological studies on a number of previously described provisional *Shigella* serotypes are reported. On the basis of the results obtained it is suggested that provisional serotypes 58 and 2050-52 may now be added to *Shigella* subgroup A as *Shigella dysenteriae* 9 and *S. dysenteriae* 10, respectively, and that serotypes 123, 425, 2770-51, and 703 may be added to *Shigella* subgroup C as *Shigella boydii* 12, *S. boydii* 13, *S. boydii* 14, and *S. boydii* 15, respectively. It is suggested that the status of serotypes 3873-50, 3615-53, and 1621-54 remain provisional for the present. Also, the biochemical and serological relationships of three new provisional *Shigella* serotypes are reported. These are serotypes 2000-53, 3341-55, and 2710-54.

The status of several other serotypes, some of which were previously described as provisional shigellae, have been investigated and discussed and it is concluded that these are not shigellae but anaerogenic *Escherichia coli* instead.

Introduction

In 1953 the International Enterobacteriaceae Subcommittee recommended the addition of several serotypes to the shigella schema (9). Accordingly, these serotypes were added to the schema as *Shigella dysenteriae* 8, *Shigella boydii* 8, *S. boydii* 9, *S. boydii* 10, and *S. boydii* 11. The Subcommittee also recommended that certain other described *Shigella* serotypes be considered for possible future addition to the shigella schema but remain *sub judice*. These were serotypes 58, 3873-50, 2050-52, 425, 703, 2770-51, and 123. The purpose of this paper is to report the results of continued biochemical and serological studies with cultures belonging to the above-mentioned provisional serotypes, to characterize three undescribed serotypes, and to consider the taxonomic position of each. Also the results of studies with a group of anaerogenic *Escherichia coli* cultures, which resembled shigellae in some respects, are reported and the taxonomic position of such strains is discussed.

Methods

The sources of the group of strains included in the investigation were given in Table I. The methods used in the study of the biochemical and serological activities of the cultures were similar to those recommended by Kauffman (23), Edwards and Ewing (7), and Ewing *et al.* (18).* Lysine decarboxylation was determined by the ninhydrin method, details of which were given by Carlquist (1) and by Edwards *et al.* (8). Leifson's malonate broth with added yeast extract was used for determination of malonate utilization and tests for the production of phenylpyruvic acid (PPA) from phenylalanine (PA) were made by the PA agar method (19).

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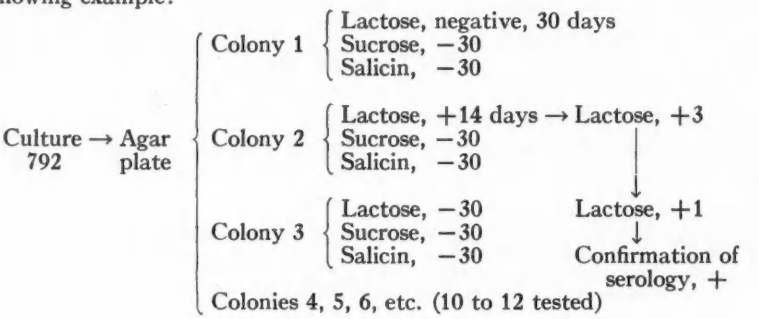
Contribution from the Communicable Disease Center, Public Health Service, U.S. Department of Health, Education, and Welfare, Atlanta, Georgia.
[2]International Shigella Center, Atlanta, Georgia.
*A mimeographed outline of biochemical methods is available upon request.

TABLE I

SOURCES OF SEROTYPES

Serotype	No. of cultures	Case	Carrier	Unknown
		Previously described provisional *Shigella* serotypes		
58	4	2		2
3873-50	2	1		1
2050-52	3	3		
3615-53	4	3		1
123	4	3	1	
1621-54	5	4		1
425	10	8	2	
2770-51	12	12		
703	7	4	2	1
		New provisional *Shigella* serotypes		
2000-53	1		1	
3341-55	1			1
2710-54	2	2		
		Other anaerogenic serotypes		
2044-54	2	2		
3617-53	10	7	1	2
H62	3	3		
1831	9	1	3	4 (1, flies)
792	3	2	1	

The method employed in the study of mutative fermentation of various carbohydrates was that used earlier in connection with mannitol fermentation by *Shigella flexneri* strains (16). Cultures were plated on infusion agar medium and portions of each colony selected were transferred to individual tubes of lactose, sucrose, and salicin broths. In many instances mannitol and raffinose tubes also were inoculated. Ten to 12 colonies from each culture were inoculated into the carbohydrate media. After inoculation, the tubes were sealed with cork stoppers, which had been soaked in hot paraffin, and incubated at 37 °C. When fermentation occurred in one of the tubes, serial transfers were made in tubes containing the carbohydrate that was fermented. At the end of such experiments the serology of the variant was confirmed. Negative tests were observed for 30 days. The method may be illustrated by the following example:



Studies on mutative fermentation of certain carbohydrates were made with all of the cultures belonging to the serotypes listed in Table I and with cultures of recognized *Shigella* serotypes as well.

Observations

Previously Described Provisional Shigella Serotypes

Serotype 58

This serotype was described by Cox and Wallace (5), who mentioned a single strain, which was isolated in the India–Burma area during the second World War. This culture was received from Dr. C. D. Cox in 1949. More recently three additional strains of serotype 58 became available to the writers. The first of these (3307-53, Palermo 844) was recovered from the stool of patient with dysentery in Sicily and was received from Dr. D'Alessandro of Palermo. The second strain (2549-55) was received from Mr. E. K. Borman of the Connecticut State Health Department Laboratories, who informed the writers that the culture had been isolated from a patient who had contracted dysentery while visiting Mexico. The last strain was isolated in Guatemala during a survey and was sent to the writers by Dr. A. S. Browne of the California State Department of Health Laboratories.

The biochemical reactions given by the above-mentioned serotype 58 strains (Table II) were similar to those given by members of *Shigella* subgroup A (*S. dysenteriae*), which contains the shigellae that characteristically do not produce acid from mannitol. Mutative fermentation of lactose, sucrose, or salicin, was not demonstrated in any of the serotype 58 cultures.

TABLE II

BIOCHEMICAL REACTIONS OF PREVIOUSLY DESCRIBED AND NEW PROVISIONAL *Shigella* SEROTYPES

	Previously described serotypes									New serotypes		
	58	3873-50	2050-52	3615-53	123	1621-54	425	2770-51	703	2000-53	3341-55	2710-54
Sucrose	−	−	−	−	−	x	−	−	−	−	−	−
Mannitol	−	−	−	+	+	+	+	+*	+	−	−	+
Dulcitol	−	−	−	−	−orx	−	−	−	−	−	−	−
Rhamnose	−	−	−	−	−	−orx	−	−	−	+	+	−
Arabinose	+	+	+	+	+	−orx	+	+	x	+	x	+
Xylose	−orx	x	+	+	−	−	−	d	−	−	x	+
Sorbitol	−orx	x	+	+	−orx	−orx	+	+	+	−	+	+
Trehalose	+	+	d	−	+	+	+	+	+	+	−	d
Maltose	−orx	x	−	−orx	−orx	+	−	d	d	+	−	x
Raffinose	−	−	−	−	−	x	−	−	−	−	−	d
Indole	−	−	−	+	−	+	+	−	+	+	−	+
Ammonium salts glucose agar	−orx	x	+orx	−	+orx	−	d	d	d	−	x	+

NOTE: All available strains of all of the serotypes listed in Table II fermented glucose without gas production, all produced nitrite from nitrate, and all gave positive methyl red tests. All of the cultures failed to produce acid from lactose, salicin, adonitol, and inositol, were Voges-Proskauer negative, and failed to utilize citrate in either Simmons' or Christensen's citrate medium. All strains gave negative results in tests for gelatin liquefaction, hydrogen sulphide production, mucate and malonate utilization, urease and phenylalanine deaminase production, all failed to grow in KCN medium, and all gave negative results in the ninhydrin test of Carlquist (1956). All of the cultures were nonmotile.

*Occasional strains failed to ferment mannitol (see text). + = Positive 1 or 2 days; x, positive slowly (3 or more days); −orx, some strains negative, some strains positive slowly, but none positive in 1 or 2 days; d, some strains +, some strains x, and some strains −; −, no reaction.

No significant serological relationship was noted between serotype 58 strains and recognized *Shigella* serotypes or any of the serotypes mentioned herein. Also, no significant relationship was detected between the O antigens of the 137 *E. coli* O groups and those of serotype 58 strains. The four cultures all possessed the same K antigen, which was of the B variety. Their K antigens appeared to be unrelated to those of other shigellae.

It is believed that serotype 58 may now be added to *Shigella* subgroup A as *S. dysenteriae* 9.

Serotype 3873-50

Two cultures of this serotype were described by Ewing and Hucks (13). One of these was recovered from a patient with dysentery but the source of the other strain was unknown. The two serotype 3873-50 strains produced acid from xylose, sorbitol, and maltose upon extended incubation (6 to 30 days) and both were able to grow on ammonium salts glucose agar after 6 days' incubation. The remaining biochemical reactions given by the two cultures were uniform (Table II). Further, mutative fermentation of lactose, sucrose, or salicin was not demonstrated in either of the strains.

The O antigens of serotype 3873-50 cultures were not significantly related to those of recognized shigellae, nor to those of the various serotypes mentioned in this report. However, the O antigens of cultures of this serotype were related to those of *E. coli* O group 29 (13). The K antigens of this serotype have not been examined in detail.

The biochemical reactions given by serotype 3873-50 cultures were similar to those given by recognized types included in subgroup A of the shigella schema and it is believed that continued consideration should be given them regarding possible future addition to that subgroup. However, no additional strains of the serotype have been received since 1950 and it is believed that this serotype should remain *sub judice* for the present. It may be referred to as provisional serotype 3873-50, as in the past.

Serotype 2050-52

The type culture of this serotype was received in 1952 from Dr. J. Vandepitte, who isolated the strain from the stool of a dysenteric patient in the Belgian Congo. This strain was described by Ewing (15), who reported that except for a relatively minor O antigenic relationship to *S. dysenteriae* 2, serotype 2050-52 was not related to other shigellae. Two additional strains of this serotype were identified by Dr. K. P. Carpenter of the International Shigella Center, London, in 1956. Dr. Carpenter stated that the cultures were isolated by Mr. W. G. Tarrant from patients with dysentery in Northern Rhodesia.

Mutative fermentation of lactose or salicin was not demonstrated in serotype 2050-52 cultures, but one colony (colony 5) isolated from strain 2050-52 produced slight acidity in sucrose broth after 3 days' incubation. After four passages in sucrose broth the progeny of colony 5 produced acid from this substance within 1 day. Mutative fermentation of sucrose was not detected

in the other two, more recently isolated, serotype 2050-52 cultures. Strain 2050-52 produced acid from trehalose within 1 day but the other two cultures failed to ferment it. The remaining biochemical reactions given by the three cultures were identical (Table II).

The O antigens of serotype 2050-52 cultures were not significantly related to those of any of the 137 recognized *E. coli* O antigen groups. However, a relationship was detected between the O antigens of serotype 2050-52 strains and culture 1624-56 (Table III). The latter was an anaerogenic *E. coli* culture, which apparently represented a new O antigen group, since its O antigens were not significantly related to those of any of the 137 *E. coli* O antigen groups.

TABLE III

THE O ANTIGENIC RELATIONSHIP OF SEROTYPE 2050-52 STRAINS AND *E. coli* 1624-56

O antigen suspensions (100° C., 1 hr.)	O antisera			
	Serotype 2050-52		*E. coli* 1624-56	
	Unabsorbed	Absorbed by 1624-56	Unabsorbed	Absorbed by 2050-52
Serotype 2050-52	2,560*	1,280	160	0
E. coli 1624-56	2,560	0†	10,240	5,120

*Reciprocal of the highest dilution that gave strong agglutination.
†Negative in dilutions of 1:40 or higher.

The presence of K antigens was demonstrated in serotype 2050-52 strains and these antigens were not related to those of other shigellae. However, the nature of the K antigens of this serotype has not been determined.

Although mutative fermentation of sucrose was demonstrated in one of the three serotype 2050-52 cultures, this was not regarded as sufficient reason to reject the serotype from the *Shigella* group, since certain *S. flexneri* and *S. dysenteriae* cultures sometimes produce acid from sucrose slowly, especially after they have been cultivated on artificial media for some time. The biochemical reactions of serotype 2050-52 strains were similar to those given by members of *Shigella* subgroup A and the cultures possessed a type antigen by which they may be identified and differentiated from other shigellae; hence, it was believed that serotype 2050-52 may be added to the shigella schema as *S. dysenteriae* 10.

Serotype 3615-53

Three cultures of this serotype were received during 1953 and 1954 from Dr. Kirsche, then of Hanoi, who stated that the strains were isolated from the stools of patients with dysentery in Indochina. The three cultures were related to *S. dysenteriae* 3 but were indole-positive and fermented mannitol. The biochemical reactions given by the three strains (Table II) were uniform

with exception of their reactions in maltose medium. One culture (3615-53) produced acid from maltose after 7 days' incubation, while the other two failed to utilize this substrate. Mutative fermentation of lactose, sucrose, or salicin was not demonstrated. During 1956 a culture (5519-56) was received from Dr. K. P. Carpenter, who stated that the strain produced acid from mannitol and failed to form indole, and that its O antigens were identical with those of *S. dysenteriae* 3. The writers confirmed Dr. Carpenter's findings with culture 5519-56 (Carpenter 196/53) in all respects and regarded it as a mannitol-positive variety of *S. dysenteriae* 3.

The O antigenic relationship of serotype 3615-53 cultures to *S. dysenteriae* 3 and to *E. coli* O group 124 were reported earlier (15, 18). The presence of K antigen was demonstrated in serotype 3615-53 strains but the nature of their K antigen has not been determined. However, it was established that the K antigen of serotype 3615-53 was not related to that of *S. dysenteriae* 3. The O and K antigens of serotype 3615-53 did not appear to be significantly related to those of other shigellae.

There can be little doubt about the taxonomic position of culture 5519-56 (Carpenter 196-53). The O and K antigens of this strain were identical with those of *S. dysenteriae* 3; hence, it appeared that it must be regarded as a mannitol-positive variety of that serotype. However, the taxonomic position of serotype 3615-53 may be debated, since cultures of this serotype were not antigenically identical with *S. dysenteriae* 3. At least three possible solutions to this problem may be presented:

(*a*) serotype 3615-53 might be added to *Shigella* subgroup C, in which case it would be considered a new *S. boydii* serotype having an intersubgroup antigenic relationship with *S. dysenteriae* 3;

(*b*) serotype 3615-53 might be considered as a subserotype of *S. dysenteriae* 3 since the O antigens of these two were intimately related; or

(*c*) serotype 3615-53 might be considered as a separate serotype within *Shigella* subgroup A and having intrasubgroup antigenic relationship with *S. dysenteriae* 3.

Of these possible solutions, the writers preferred the first (*a*), in spite of the fact the O antigens of serotype 3615-53 were closely related to those of *S. dysenteriae* 3. As stated above, the K antigens of the former serotype were not related to those of the latter. Also, it was believed that *new* serotypes that produce acid from mannitol should not be admitted to *Shigella* subgroup A if it can be avoided. This solution may be considered somewhat arbitrary but it seemed less arbitrary than to admit a mannitol-positive serotype to subgroup A, either as a subserotype of an existing serotype or as a separate serotype, since solutions (*b*) and (*c*) above both were contrary to existing concepts regarding the composition of the several subgroups of *Shigella*. It is one thing to recognize the existence of an occasional mannitol-positive variety of a member of subgroups B, C, or D, and quite another to admit a mannitol-positive serotype to subgroup A, which is composed of serotypes and biotypes that *characteristically* are mannitol-negative, and which

have been recognized as being so for many years. Furthermore, the writers were of the opinion that, insofar as it is possible to do so, serotypes having different K antigens should be kept separated, even though their O antigens may be intimately related. This may be accomplished easily with serotypes that belong to *Shigella* subgroups A, C, and D because subserotypes have not been officially recognized or designated within these subgroups.

It was believed that serotype 3615-53 should be considered for possible addition to the *S. boydii* subgroup but that its status should remain provisional for the time being.

Serotype 123

The type culture of this serotype was isolated in Italy in 1943 and was described by Ewing and Hucks (13). Two other strains belonging to serotype 123 were studied extensively by other investigators as well as by the writers. These were the type originally designated M (1693), which was isolated by Gildemeister in Finland during 1943, and culture 7*a* (1386-50) from Sicily (2, 4, 13, 21, 28). More recently, a fourth strain (1415-56) was received by the writers. This culture was isolated at the National Institutes of Health, Bethesda, Maryland, from the stool of a monkey that was ill with dysentery.

The biochemical reactions given by serotype 123 strains (Table II) were similar to those given by members of the *S. boydii* subgroup. One culture (1415-56) produced acid from dulcitol, sorbitol, and maltose after 2 to 14 days while the remaining strains failed to utilize these substrates. Otherwise, the reactions given by the four cultures were identical. Mutative fermentation of lactose or salicin was not demonstrated in any of the strains. However, weak transient acidity was produced in sucrose broth by the progeny of some colonies isolated from all four serotype 123 cultures. These weak reactions were not intensified by serial passage in sucrose medium.

The O antigens of serotype 123 cultures were divisible into at least three fractions and one of these was shared by *E. coli* O group 7 and Alkalescens-Dispar (A-D) O group 7 strains (13). The O antigens of serotype 123 strains did not appear to be significantly related to those of other shigellae although relatively minor relationships to several *S. flexneri* serotypes (e.g. *S. flexneri* 6) were noted. Also, no relationship was detected when living suspensions of serotype 123 cultures were tested in K antisera prepared with other *Shigella* serotypes. However, studies on the K antigens of serotype 123 cultures are incomplete. For example, it is not known with certainty whether the M culture possesses a K antigen or, if it does, whether its K antigen is identical with that of culture 123.

It is believed that serotype 123 now may be added to *Shigella* subgroup C as *S. boydii* 12. The M variety may be included as a variant of *S. boydii* 12 for the present but it may be desirable to separate it in the future.

Serotype 1621-54

Cultures belonging to this serotype were mentioned by Sahab (27), who reported the isolation of four strains from patients with dysentery in Indonesia.

These cultures were received from Dr. K. Sahab in 1954 and a fifth strain was received from Dr. Kirsche, also in 1954.

The biochemical reactions (Table II) given by serotype 1621-54 cultures were similar to those given by members of the *S. boydii* subgroup except that raffinose was fermented mutatively by three of the four strains and the fourth utilized it within 1 day. Mutative fermentation of sucrose was demonstrated in all of the cultures but mutative fermentation of lactose or salicin was not noted. One strain produced acid from rhamnose after 13 days and another fermented arabinose after 7 days' incubation, while the other four cultures failed to produce acid from either of these substances. Two cultures failed to ferment sorbitol and three required from 3 to 13 days for acid production. One strain required 7 days' incubation before acid production from maltose became apparent while the remaining cultures utilized this substrate within 1 day. The results of other biochemical tests were uniform (Table II).

Raffinose utilization on the part of serotype 1621-54 cultures tended to eliminate them from the *S. boydii* subgroup, since it was known that recognized *S. boydii* types did not produce acid from this substrate whereas the majority of *S. flexneri* types did (26). Parenthetically, it may be added that serotype 1621-54 cultures produced a color reaction in medium that contained phenylproprionic acid. This result also tended to eliminate serotype 1621-54 from consideration as a member of the *S. boydii* subgroup since it appeared that most of the *S. boydii* cultures failed to give this reaction while a majority of members of the *S. flexneri* subgroup did so (6). However, admission of serotype 1621-54 to the *S. flexneri* subgroup did not seem justified because no significant antigenic relationship was detected between the O antigens of serotype 1621-54 cultures and those of *S. flexneri* strains. Thus, it seemed that if serotype 1621-54 were admitted to the *Shigella* group, it would have to be added to subgroup C as an exceptional, raffinose-positive, phenylproprionic acid positive type.

The results of reciprocal agglutinin absorption tests revealed that the O antigens of serotype 1621-54 cultures were identical with those of *E. coli* O group 7 and A-D O7 and that serotype 1621-54 was related to serotype 123 in the same manner as *E. coli* O7 and A-D O7. Since living suspensions prepared with the serotype 1621-54 were agglutinated in O antiserum, the presence of K antigens in them has not been established.

Since serotype 1621-54 was serologically related to serotype 123, the former might be considered a subserotype of the latter. However, known cultures of serotype 123 did not ferment raffinose, did not produce a color reaction in phenylproprionic acid medium, and did not form indole while serotype 1621-54 strains gave positive results in all of these tests. Hence, it is believed that serotype 1621-54 should be regarded as a separate serotype and biotype and that it should remain *sub judice* until further consideration can be given to its taxonomic position.

Serotype 425

Ten strains of this serotype have been collected and studied over the past several years. Three of these were isolated in Italy and Belgian Congo (13), five were isolated in Egypt by Commander T. M. Floyd and associates and were sent to the writers by Commander L. A. Barnes of the Naval Medical Research Institute, Bethesda, Maryland, and the last two originated in Florida (Table I).

Except for differences in the ability of individual strains to grow on ammonium salts glucose agar, the biochemical reactions of the above-mentioned 10 serotype 425 strains were identical (Table II). Six cultures failed to grow on ammonium salts glucose agar, while three produced positive reactions in 1 day, and one strain yielded a positive test after 2 days' incubation. Mutative fermentation of lactose, sucrose, or salicin was not observed in serotype 425 cultures.

Ewing and Hucks (13) reported that the O antigens of serotype 425 cultures were related to those of *S. boydii* 9 and recommended reciprocal absorption of diagnostic antisera for these two serotypes. These workers also reported that the O antigens of serotype 425 strains were related to those of *E. coli* O antigen groups 28 and 98.

Since the biochemical reactions of serotype 425 cultures were similar to those given by the shigellae of subgroup C, and since strains belonging to this serotype can be distinguished readily from other shigellae, it is believed that serotype 425 may be added to subgroup C as *S. boydii* 13.

Serotype 2770-51

Since the type culture of this serotype was described (13), 11 additional strains have been received and studied. Five of these originated in a familial outbreak and all were isolated from the stools of patients with dysentery in the United States.

The biochemical reactions given by serotype 2770-51 cultures (Table II) were similar to those given by members of *Shigella* subgroup C. Mutative fermentation of lactose, sucrose, or salicin was not demonstrated with strains of this serotype. Two strains, 3436-53 and the epidemic strain, represented by five cultures from a familial outbreak, failed to produce acid from mannitol but were similar to the type culture in other respects. One strain failed to ferment xylose, one fermented it within 1 day, and five produced acid from this substrate after 4 to 10 days' incubation. Four cultures failed to produce acid from maltose, two fermented it within 1 day, and one required 9 days to produce a positive reaction. Three strains failed to grow on ammonium salts glucose agar, while four did so within 1 or 2 days. With these exceptions, the seven cultures of serotype 2770-51 yielded identical reactions in each of the tests listed in Table II. Attention should be directed to a typographical error in the table of biochemical reactions given by Ewing and Hucks (15). In that table serotype 2770-51 was recorded as indole-positive, whereas it should have been listed as indole-negative.

The O antigens of serotype 2770-51 cultures were not significantly related to those of other shigellae. However, it should be remembered that their O antigens are identical with those of *E. coli* O group 32 and reciprocally related to those of *E. coli* O group 83 (13). The K antigens of serotype 2770-51 cultures have not been thoroughly investigated.

The data presented indicated that serotype 2770-51 may be recommended for addition to *Shigella* subgroup C as *S. boydii* 14.

Serotype 703

The type culture of this serotype, isolated in Italy in 1944, was described by Ewing, Hucks, and Taylor (14). Since then three other strains of the serotype were identified among cultures isolated in Italy during the period 1943 to 1945 (10); two cultures were received from Dr. K. P. Carpenter in 1956, and one, isolated in Guatemala, was received via the California State Department of Health Laboratories in 1957. Other available information regarding the sources of the cultures was summarized in Table I.

The biochemical reactions of serotype 703 cultures (Table II) were similar to those given by members of *Shigella* subgroup C. With the following exceptions, the reactions given by the seven strains were identical: One culture produced acid from arabinose within 1 day while the others required 5 to 7 days to ferment it; one strain fermented maltose rapidly, one required 7 days, and five failed to produce acid from maltose; and three cultures grew readily on ammonium salts glucose agar, three required 3 days' incubation before growth appeared, and one strain failed to grow on this medium. Mutative fermentation of lactose, sucrose, or salicin was not demonstrated.

The O antigens of serotype 703 cultures were identical with those of *E. coli* O 112*a*,112*b*:K68(B13):H18 (14) and are related to those of *S. dysenteriae* 2 as demonstrated by reciprocal absorption tests (Table IV). Available strains of serotype 703 all possessed the same K (B) antigen, which was closely related to *E. coli* K antigen 68 (17, 18). No significant relationship was noted between the B antigens of *S. dysenteriae* 2 and those of serotype 703.

The situation regarding the taxonomic position of serotype 703 was more or less analogous to that of serotype 3615-53, discussed above. Conceivably,

TABLE IV

THE RELATIONSHIP OF THE O ANTIGENS OF SEROTYPE 703 AND *S. dysenteriae* 2 CULTURES

O antigen suspensions (100° C., 1 hr.)	O antisera			
	S. dysenteriae 2		Serotype 703	
	Unabsorbed	Absorbed by 703	Unabsorbed	Absorbed by *S. dysenteriae* 2
S. dysenteriae 2	10,240	5,120	640	0
Serotype 703	640	0	10,240	2,560

serotype 703 might be regarded as a subserotype and biotype within *S. dysenteriae* 2. However, there was less reason for such a step than there was for regarding serotype 3615-53 as a subserotype of *S. dysenteriae* 3, since the O antigenic relationship between serotype 703 and *S. dysenteriae* 2 was less extensive than that extant between serotype 3615-53 and *S. dysenteriae* 3. Further, no K antigenic relationship was detected between serotype 703 and *S. dysenteriae* 2. Hence, it was believed that the better solution to this problem was to admit serotype 703 to *Shigella* subgroup C as *S. boydii* 15, a serotype having an intersubgroup relationship with a member of *Shigella* subgroup A.

New Provisional Shigella Serotypes

Serotype 2000-53

One culture of this serotype, isolated from a carrier in Minnesota, was available for study. The biochemical reactions of this strain (Table II) were similar to those given by members of *Shigella* subgroup A. Also, it may be recorded that mutative fermentation of lactose, sucrose, or salicin was not demonstrated in the serotype 2000-53 culture.

The O antigens of serotype 2000-53 were not significantly related to those of other shigellae. However, the results of reciprocal agglutinin absorption studies indicated that the O antigens of this serotype were identical with those of *E. coli* O group 6 and unilaterally related to those of *E. coli* O group 57. The K antigens of serotype 2000-53 have not been investigated.

Serotype 2000-53 may be considered for possible future addition to *Shigella* subgroup A, but it is believed that its status should remain provisional for the present.

Serotype 3341-55

This serotype was isolated in Arizona but details regarding the history of the strain were unavailable. The biochemical reactions of the single serotype 3341-55 culture presently available for study were similar to those given by members of *Shigella* subgroup A (Table II). Mutative fermentation of lactose, sucrose, or salicin was not noted.

The O antigens of serotype 3341-55 were not significantly related to those of other shigellae, but they were related to those of *E. coli* O3, as demonstrated by reciprocal absorption tests. Both the serotype 3341-55 and *E. coli* O3 cultures possessed a specific O antigen fraction that may be identified by the use of cross-absorbed antisera. The K antigens of serotype 3341-55 have not been investigated in detail.

It appeared that serotype 3341-55 may be considered for possible future addition to *Shigella* subgroup A, but its status should be regarded as provisional.

Serotype 2710-54

The type culture of this serotype was isolated from the stool of a patient with dysentery in Indochina and was sent to the writers by Dr. Kirsche. A second strain (5518-56) was received from Dr. K. P. Carpenter, who stated that it

had been isolated from another such patient in Hong Kong. The biochemical reactions given by the two serotype 2710-54 cultures (Table II) were uniform and were similar to those given by members of *Shigella* subgroup C. Mutative fermentation of lactose, sucrose, or salicin was not demonstrated.

The O antigens of serotype 2710-54 strains were not significantly related to those of other shigellae. However, the results of reciprocal agglutinin absorption tests indicated that their O antigens were related to those of *E. coli* O group 41. Serotype 2710-54 and *E. coli* O group 41 cultures each possessed a specific O antigenic fraction. The K antigens of serotype 2710-54 cultures have not been studied.

It is believed that serotype 2710-54 may be considered for possible addition to *Shigella* subgroup C in the future, but its status should remain provisional at present.

Other Anaerogenic Serotypes

The biochemical reactions of several anaerogenic strains are recorded in Table V. Some of these have been considered to be shigellae and a discussion of such microorganisms was included in the present paper in order to demonstrate the manner in which many cultures that are more or less intermediate between the *Shigella* and typical *E. coli* may be excluded from the *Shigella* group, and to illustrate certain principles regarding the taxonomy of shigellae.

TABLE V

THE BIOCHEMICAL REACTIONS OF CERTAIN ANAEROGENIC BIOTYPES AND SEROTYPES

	2044–54				H62			1831				792		
	2043-54	2044-54	6275-52	147	H62	2715-54	2716-54	1831	6a	2110-55	1626-56	792	948	439-55
Lactose	−	−	−	−orx	−	−	−	−	−	−	−	−*	−	x
Sucrose	−	−	−	−orx	−	−	−	−	−	−	−	−	−	−
Mannitol	+	+	+	+	−	−	x	−	+	+	+	+	+	+
Dulcitol	−	−	−	−	−	−	−	−	−	x	+	−	−	−
Rhamnose	−	−	+	+	+	+	+	−	−	−	−	+	+	+
Xylose	−	−	+	+	−	−	−	+	−	x	−	+	+	+
Sorbitol	−	−	+	+	x	+	x	−	−	+	+	+	+	+
Maltose	+	+	+	+	+	−	−	+	+	+	+	+	+	+
Salicin	−*	−	−	−	−	−	−	−	x	−	−	−	−	−
Raffinose	−	−	−	−	+	−	−	−	−	−	−	x	−	−
Christensen's citrate	−	−	−	d	−	−	+	x	−	−	−	−	−	−
Ninhydrin test (Carlquist)	−	−	−	−	−	+	+	−	−	−	−	−	−	−
Ammonium salts glucose agar	−	−	+	d	+	−	−	+	+	+	+	−	−	+
Mucate	−	−	+	−	−	−	−	−	+	+	+	−	−	−

NOTE: Available strains of all of the serotypes listed in Table V fermented glucose, arabinose, and trehalose without production of gas, all produced nitrite from nitrate, all except one serotype 147 strain formed indole, and all gave positive methyl red tests. All of the cultures failed to produce acid from adonitol or inositol, were Voges-Proskauer negative, and failed to utilize citrate in Simmons' medium. All strains gave negative results in tests for gelatin liquefaction, hydrogen sulphide production, malonate utilization, urease and phenylalanine deaminase production, all failed to grow in KCN medium, and all were nonmotile.

*Mutative fermentation demonstrated (see text).

Serotype 2044-54

Two strains of this serotype were isolated by Commander Floyd and associates in Egypt and were forwarded to us by Commander L. A. Barnes. The two cultures were recovered from native children who were ill with dysentery. The biochemical reactions of the two serotype 2044-54 cultures (Table V) were similar to those given by *Shigella* subgroup C cultures and mutative fermentation of lactose or sucrose was not demonstrated. However, mutative fermentation of salicin occurred in one of the strains (2043-54) but not in the other.

Culture 2044-54 cannot be excluded from the *Shigella* group by methods presently available and if additional strains that give biochemical reactions similar to those given by culture 2044-54 are found it may become desirable to add this serotype to *Shigella* subgroup C. The fact that a closely related strain (2043-54) fermented salicin would not preclude this possibility. For the present, however, it is believed that serotype 2044-54 should remain *sub judice.*

The O antigens of serotype 2044-54 cultures were not significantly related to those of shigellae but their O antigens were related to those of *E. coli* O group 8, as demonstrated by reciprocal agglutinin absorption tests. The serotype 2044-54 and *E. coli* O group 8 cultures each possessed a specific O antigenic component by which they may be identified.

Serotype 6275-52

The biochemical activity of culture 6275-52 (Table V) was too extensive for it to be considered as a member of the *Shigella* group. The strain produced acid promptly from rhamnose, xylose, sorbitol, and maltose, which was characteristic of members of the *E. coli* group rather than shigellae. The fact that culture 6275-52 also utilized mucate provided ancillary evidence in favor of the exclusion of this type from the *Shigella* group, since shigellae are not known to utilize this substrate (23, 24). The O antigens of serotype 6275-52 were not significantly related to those of shigellae or those of any of the 137 *E. coli* O antigen groups. The writers classified the strain as an anaerogenic *E. coli*, O group undetermined.

Serotype 147

Ten strains of this serotype were available for study. The type culture was isolated in Italy in 1944 and nine additional strains were collected since that time. Four of the cultures were recovered from the stools of as many cases of diarrheal disease in England and elsewhere and were sent to the writers in 1956 by Dr. K. P. Carpenter (Carpenter type 145-46). The biochemical reactions of the serotype 147 strains are summarized in Table V. One strain (904) produced acid from lactose after 3 days and one (152) fermented sucrose after 7 days, while the remaining cultures did not utilize either of these substrates. One culture (3617-53) was tested for mutative fermentation of lactose, sucrose, and salicin but such was not demonstrated by the method used. The behavior of the 10 strains with respect to their utilization of other carbohydrates was quite uniform. One culture (5524-56) failed to form detectable amounts

of indole, four failed to grow on ammonium salts glucose agar, and three alkalinized Christensen's citrate agar. One strain gave a positive ninhydrin test. Thus, serotype 147 cultures may be excluded from the *Shigella* group on the basis of their extensive biochemical reactivities (Table V), which were like those of *E. coli* rather than shigellae. Further, several strains could be excluded because of their alkalinization of Christensen's citrate agar or by means of a positive ninhydrin test.

The O antigens of serotype 147 cultures were related to those of *E. coli* O group 25 (and A-D O group 2) but were not identical with them. In fact, the serotype 147 cultures represented one of several sub-O-groups within *E. coli* O group 25 (Ewing and Davis, unpublished data). Several of the strains were O agglutinable and K antigen could not be demonstrated in them with certainty. However, at least one culture (1058) possessed *E. coli* K antigen 1 (L). Cultures of serotype 147 were identified as anaerogenic *Escherichia* on the basis of their biochemical activities and as members of *E. coli* O antigen group 25 by means of their serological reactions.

Serotype H62

This serotype was described by Piéchaud and Rubensten (25), who studied eight cultures that had been isolated from cases of dysentery in Indochina. These investigators reported that serotype H62 might be considered as a new serotype of *S. dysenteriae* (i.e. *Shigella* subgroup A). The writers received one of the H62 strains from Dr. Szturm-Rubensten and subsequently received two similar cultures from Dr. Kirsche of Hanoi. The biochemical reactions obtained with these three strains are recorded in Table V. Strain H62 produced acid from raffinose, which would tend to eliminate it from subgroups A and C, the other two cultures gave positive ninhydrin tests, and one strain (2716-53) alkalinized Christensen's citrate agar. Mutative fermentation of lactose, sucrose, or salicin was not demonstrated in strain H62.

The results of reciprocal agglutinin absorption tests indicated that the O antigens of serotype H62 cultures were identical with those of the type strain of A-D O2 (strain A-193). Thus, the O antigens of serotypes H62 and 147 (above), A-D O2, and the standard culture of *E. coli* O25 all were related and comprised three of four known sub-O-groups within *E. coli* O antigen group 25. Further, serotype H62 cultures possessed *E. coli* K antigen 1 (L), an antigen possessed by most other cultures of the A-D O2 serotype.

While the biochemical reactions of strain H62 approximated those given by typical shigellae, other cultures of the serotype differed from shigellae in that they gave positive ninhydrin tests, alkalinized Christensen's citrate agar, or were overly active in their over-all biochemical reactivities. Hence, the writers regarded the serotype H62 strains as mannitol-negative varieties of A-D O2 or *E. coli* O group 25.

Serotype 1831

This serotype was described by Wheeler and Stuart (31), who mentioned that it appeared to be a new mannitol-negative *Shigella* type. Subsequently, it was learned that culture 1831 alkalinized Christensen's citrate agar medium

and for this reason it was regarded as an intermediate coliform by Ewing and Hucks (11). These workers also reported that the O antigens of strain 1831 were identical with those of *E. coli* O group 22. Culture 6*a* was received from Dr. D'Alessandro of Palermo, Sicily, in 1950. This strain, which fermented salicin in the writers' hands, was found to be antigenically identical with culture 1831. Additional strains (Table I) have since been received and the biochemical reactions of representative cultures were recorded in Table V. It may be noted that in Table V the reaction of strain 6*a* in salicin broth was recorded as x, which meant that acid production was delayed. When examined by the method given above, it was learned that salicin fermentation by strain 6*a* probably was mutative in character. Ten colonies were picked to individual tubes of salicin broth and the progeny of several colonies produced acid in 8 to 11 days, while the progeny of other colonies failed to utilize the substrate. Serial transfer of inocula from positive tubes in salicin medium produced variants that utilized salicin within 1 day. As noted above, strain 6*a* fermented salicin slowly when first received by the writers in 1950. However, Dr. D'Alessandro reported (personal communication, 1950) that the culture did not produce acid from salicin (see also 22). Seeliger (28) mentioned that strain 6*a* did not utilize salicin when it was first examined but did so when reexamined a year later. These apparent discrepancies in the literature regarding salicin fermentation by strain 6*a* were readily explainable in view of results of the studies on mutative fermentation outlined above. Mutative fermentation of salicin was not demonstrated in culture 1831 (Wheeler) although the progeny of several colonies produced slightly acid reactions after 7 or 8 days' incubation.

Serotype 1831 cultures, including strain 6*a*, may be excluded from the *Shigella* group on the basis of their alkalinization of Christensen's citrate agar, by utilization of sodium mucate, by salicin fermentation, or by a combination of these and other biochemical reactions. The writers regarded these cultures as anaerogenic biotypes of *E. coli* O group 22 and it was believed that no other name for them was either necessary or desirable.

Serotype 792

This serotype was described by Cefalu and Gulotti (3), who proposed its addition to the *S. boydii* subgroup as a provisional serotype. Strain 948 was isolated from a carrier in Italy by the senior author in 1945 and culture 439-55 was recovered from the stool of a monkey which had diarrhea. Continued study of the biochemical reactions of serotype 792 cultures (Table V) indicated that their biochemical activities were much more extensive than those of shigellae. For example, all of the cultures produced acid promptly from rhamnose, arabinose, xylose, sorbitol, and maltose. Further, mutative fermentation of lactose was demonstrated in cultures 792 and 439-55 and one strain (792) alkalinized Christensen's citrate medium. Also, culture 792 produced acid from raffinose after 14 days' incubation.

The O and K antigens of serotype 792 cultures were identical with those of *E. coli* O 28*a*,28*c*:K73(B18) strains, the original strains of which were

TABLE VI

THE O ANTIGENIC RELATIONSHIP OF *E. coli* O GROUP 28, SEROTYPE 792, AND SEROTYPE 425 CULTURES

	O antisera						
	E. coli O 28*a*,28*b*		Serotype 792 or *E. coli* O 28*a*,28*c*			Serotype 425	
				Absorbed by			
O antigen suspensions (100° C., 1 hr.)	Unabsorbed	Absorbed by 792	Unabsorbed	*E. coli* O 28*a*,28*b*	425	Unabsorbed	Absorbed by 792
E. coli O 28*a*,28*b*	10,240	2,560	5,120	0	1,280	320	0
792 or *E. coli* O 28*a*,28*c*	5,120	0	20,480	5,120	5,120	1,280	0
Serotype 425	640	0	1,280	0	0	20,480	5,120

described by Scholtens (30) under the designation of the Katwijk type.* The O antigenic relationships extant between *E. coli* O 28*a*,28*c*:K73(B18), including serotype 792 strains, the standard *E. coli* O group 28 culture (O 28*a*,28*b*), and *Shigella* serotype 425, which was also interrelated, were recorded in Table VI. It may be noted that the relationship of serotype 792 and *E. coli* O 28*a*, 28*b* was quite extensive, as demonstrated by the extent of the agglutination reactions in unabsorbed antisera and by the degree of reduction of titer in the absorption tests. The relationship between *E. coli* O group 28 strains, including serotype 792, and *Shigella* serotype 425 was less extensive, the latter being more closely related to *E. coli* O group 98.

As mentioned above, the serotype 792 cultures may be classified as *E. coli* on the basis of their biochemical activities and their serological relationships permitted their classification as *E. coli* O 28*a*,28*c*:K73(B18).

Numerous other examples of anaerogenic, nonmotile *E. coli* biotypes that resemble shigellae in certain respects might be given, since such cultures are known to exist among members of almost all of the known 137 *E. coli* O antigen groups. However, such biotypes usually may be eliminated from consideration as possible shigellae if the whole of their biochemical reactivities are considered. The examples given above should suffice to illustrate these points.

Discussion

The family Enterobacteriaceae is composed of a number of delineated groups of bacteria, each of which consists of types that give similar biochemical reactions. Some of these groups are related to each other through the similarity of their reactions in certain rather fundamental or basic biochemical reactions and by means of close antigenic relationships. For example, the *Salmonella* and Arizona groups are related to each other in this way and it may be said that they belong to the same principal group, which might be referred to as a tribe. Similarly, the *Escherichia* and *Shigella* groups are more closely related to each other, both biochemically and serologically, than either is related to any other group and may be said to belong to the same principal group. However, it is well known that intermediate biotypes exist that may not be readily classified in one of the delineated groups, although the principal group to which such a culture belongs may be perfectly apparent. Biotypes that are more or less intermediate and near the border line between the *Shigella* and *Escherichia* groups are not uncommon, but fortunately these usually can be classified as one or the other if their biochemical reactivities are considered as a whole, as illustrated in the studies reported. In those instances where a culture cannot be definitely classified as a member of the *Shigella* group or of the *Escherichia* group, an arbitrary decision usually is made and the culture classified as a provisional member of one group or the other. Such decisions need not be considered permanent in all instances, but they should only be made after careful consideration in order to minimize the number of changes that may have to be made at a later date. In a few instances the better

*For additional references to *E. coli* O 28*a*,28*c*:K73(B18), Katwijk, see (18, 20, and 29).

solution may be to regard a particular collection of related, borderline strains as *sui generis* if they can be differentiated. Such a *sui generis* group later may become a recognized, delineated group.

The lactose-positive strains isolated in Belgian Congo by Courtois and Vandepitte (personal cummunication, 1950), may be cited as an example of the sort of borderline culture referred to above. The O antigens of these strains were identical with those of typical *S. boydii* 9 cultures (Courtois and Vandepitte, personal communication, 1950; and (12)) and except for fermentation of lactose, the biochemical reactions given by the cultures were similar to those given by *S. boydii* 9 strains. Further, the cultures cannot be excluded from the *Shigella* group by methods presently available, except on the basis of their production of acid from lactose. Since these cultures produced acid from lactose, they might be classified as *E. coli* O group 102, to which they are related serologically, or, since lactose fermentation was their only aberrant characteristic, they might be classified as aberrant, lactose-positive *S. boydii* 9 strains. Although no arbitrary decision has been made in this instance, the writers regarded this biotype as a lactose-positive variety of *S. boydii* 9 (provisional designation). It is believed that the occasional occurrence of a lactose-positive variety of an existing *Shigella* type must be recognized, provided that the other biochemical reactions given by the strain conform to the definition of the *Shigella* group. This does not mean that new types that produce acid from lactose should be indiscriminately added to the *Shigella* group. Nor does it mean that the definition of the *Shigella* group must be changed. We are merely recognizing the existence and occasional occurrence of a lactose-positive variety of an existing type, just as lactose-fermenting varieties of certain salmonellae are known and recognized. The occurrence of occasional strains of *S. dysenteriae* 1 that produce acid from lactose has been known for many years; hence, there is nothing new in the above-mentioned concept.

Studies on mutative fermentation of lactose, salicin, etc., must be continued and enlarged before their value to taxonomy can be fully assessed. However, the investigations reported herein indicated that the method was of value. When mutative fermentation of lactose or salicin was demonstrated it afforded ancillary evidence that the strain under investigation was not a member of the *Shigella* group. When this evidence was considered along with other reactions, it was possible to eliminate certain more or less borderline strains from consideration as possible shigellae.

It is believed that new serotypes should be characterized as fully as possible by means of their biochemical reactions, including studies aimed at the demonstration of mutative fermentation, and their serological reactions and relationships. Such serotypes should be described but their status should remain *sub judice* for a considerable period, during which additional strains may be collected and studied by interested workers in various parts of the world. The collection of additional cultures is of considerable importance in the determination of whether a bacterial type ought to be added to the *Shigella* group and in the determination of the subgroup to which it belongs if it is added.

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BACTERIAL PERMEABILITY: THE UPTAKE AND OXIDATION OF CITRATE BY ESCHERICHIA COLI[1]

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Abstract

A direct measurement of total uptake was applied to determine whether surface impermeability could explain why extracts of *Escherichia coli* can oxidize exogenous citrate but intact cells cannot. The equations for the "space" method are derived and its limitations are pointed out. The observed uptake of citrate was characterized as a variable that was dependent on temperature, pH, external citrate concentration, the species and concentration of cations, and integrity of the cell, but was independent of added energy source, time, and adaptational or mutational processes. Uptake into the citrate space appeared to be a diffusional process. It was concluded that citrate normally diffuses into the cell wall, and under certain environmental conditions may penetrate further into the cell. The lack of correlation between uptake and oxidation of citrate negated the hypothesis of surface impermeability of *E. coli* to citrate.

Introduction

Recent advances in the study of cellular structure and metabolism have brought renewed interest in the role of semipermeable structures located at the surface and in the interior of the bacterial cell. Relatively little has been done to establish whether permeability is a limiting factor in respiration, although the phenomenon of semipermeability repeatedly has been advanced to account for observations which otherwise seemed unexplainable. One of the most frequently reported of these observations is the case in which disrupted organisms are found to contain enzymes that cannot be demonstrated in the intact cells; a classic instance is the inability of *Escherichia coli* to oxidize or exchange endogenous citrate whereas cell extracts can do so.

Although it seemed possible to study this anomaly in *E. coli* by determining the variables that might induce citrate oxidation by intact cells, our attempts to demonstrate the involvement of citrate when added exogenously have been essentially unsuccessful. Results of this approach in the parallel case of *Brucella abortus* will be reported separately.

The present investigation was concerned with applying a direct measurement of total uptake to determine whether surface impermeability could account for the inability of intact *E. coli* to oxidize exogenous citrate and partially characterizing the citrate uptake that was in fact observed.

Materials and Methods

Measurement of the uptake of citrate was based on the concept that any cell volume which will act to dilute added substrate is permeable to that

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substrate. Therefore, the permeable fraction of the total cell volume should be measurable by the degree of this dilution when a known substrate concentration is added to a known large volume of cells. This method generally is credited to Conway and Downey (7).

When large masses of cells are packed tightly together, their more easily determined weight can be assumed to approximate their volume. However, a wet pack of cells will contain at least a little water in the interstices and this intercellular water must be allowed for in arriving at the uptake by the cells themselves. If a suspension of cells is packed to a constant weight (W_p), the pack will have a constant intercellular space (S_{in}). This intercellular space, which is defined as the fraction (or percentage) of the total volume of the cell pack that the solute can penetrate, can be determined by adding to the pack a given volume (V_s) of a known concentration (C_0) of an *impermeable* solute (in this case inulin). On suspension of the cells in this solution a certain amount of solute dilution will occur, because of the interstitial water. This will result in a final solution volume, $V_s + S_{in}W_p$, where S_{in} is equal to the interstitial volume per gram of cell pack at the final solute concentration(C_f). Thus the substrate balance after addition, mixing, and recentrifugation is:

$$\text{initial substrate} = \text{final substrate} \qquad [1]$$

or

$$C_0V_s = C_f(V_s + S_{in}W_p). \qquad [2]$$

This equation can be rewritten as an expression of the intercellular space,

$$S_{in} = V_s/W_p(C_0/C_f - 1). \qquad [3]$$

On the other hand, if the solute is one which penetrates the cells as well as the interstitial water, then the final solute concentration (C_f) will be further decreased in proportion to the amount of substrate penetrating the cells. The corresponding space value for the penetrating solute (S_{sol}) also can be determined by equation [3] but constitutes the *total* space available to the substrate per gram of cell pack and thus includes both the inter- and intracellular space. Therefore, to know how much of the cell is penetrated it is necessary to correct the S_{sol} by the S_{in}, obtaining an intracellular space (R) which is defined as the fraction (or percentage) of the volume of the cells themselves that the solute can penetrate, or

$$R = \frac{\text{penetrated cell volume}}{\text{total cell volume}}. \qquad [4]$$

The penetrated cell volume is equal to the penetrated pack volume less the intercellular volume; and the total cell volume is equal to the total pack volume less the intercellular volume. Therefore,

$$R = (S_{sol}V_p - S_{in}V_p)/(S_pV_p - S_{in}V_p). \qquad [5]$$

Since the total pack space (S_p) is of course 1.00 and since V_p (which is measured as W_p) cancels out, equation [5] is simplified to:

$$R = (S_{sol} - S_{in})/(1 - S_{in}). \qquad [6]$$

The corrected solute space (R) so determined gives the most meaningful and accurate value for uptake by the cell of a given solute and was ultimately employed in the ensuing experimental work.

In a typical experiment, cells were freshly grown and harvested, resuspended in a diluent (usually saline), dispensed into tared centrifuge tubes, and washed several times using low-speed centrifugation for repacking. After these washings the cells were packed at 20,000 g (12,500 r. p. m.) for 20 minutes in a Servall Model SS-1 angle-head centrifuge at 4° C.; this centrifugation corresponded to the plateau region on a packing curve. Then the supernatants were quickly poured off, the sides of the tubes were wiped free of water, and the tubes of packed cells were weighed to the nearest 0.1 g. Each tube contained 6 to 10 g. of packed cells (W_p). After the tubes were chilled to 0° C. an accurately measured volume of buffer, saline, or other diluent was added and the cell pack was resuspended. When the cells were thoroughly resuspended and equilibrated at 0° C., a measured volume of solute was added. This sequential addition of diluent and then solute allowed rapid and reproducible diffusion throughout the cell suspension. The total amount of liquid added (V_s) depended on the weight of the cell pack, since it was desirable to keep the V_s/W_p ratio less than 1.0 so as to minimize errors in estimating C_f. The mixture was then incubated at the desired temperature (0° C. unless otherwise stated) for 10 minutes, quickly transferred to the centrifuge, and repacked for 20 minutes at 20,000 g. Immediately after the centrifugation, the supernatant was decanted and a measured volume of it was pipetted into an equal volume of 10% trichloracetic acid. This deproteinization was necessary because a considerable amount of cellular protein, which may have contained citrate-destroying enzymes, usually was present. The solute – trichloroacetic acid solution was then appropriately diluted and analyzed.

Citrate was analyzed by a modification of the methods of Cartier and Pin (4) and Natelson, Pincus, and Lugovoy (18); the details of the modified procedure will be reported elsewhere. This modified analysis has a high degree of accuracy ($\pm 5\mu$g.) over a wide range (50 to 2000 μg. per ml.) of citrate.

The concentration of inulin was determined gravimetrically by placing the sample in tared aluminum weighing dishes and drying *in vacuo* over phosphorus pentoxide until a constant dry weight was attained.

E. coli, strain B (received from Dr. D. B. Cowie) was grown on the M-9 medium of Roberts *et al.* (21): 0.2% NH_4Cl,* 0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.5% NaCl, 0.01% $MgSO_4$, 0.8% glucose, and Dow Corning antifoam AF. Either 15 liters of the medium in a glass carboy or 30 liters in a stainless steel fermentor were used; in both cases the inoculum was 700 ml. of a stationary-phase shake-culture grown on M-9 medium at 30° C. Cells were harvested when the cultures reached a maximum turbidity. In the stainless steel fermentor the culture was mechanically agitated and aerated under an air pressure of 10 lb. per sq. in. at 34° C., and was harvested 10 hours after inoculation by means of a Sharples supercentrifuge. Cell yields varied from about

*0.1% NH_4Cl in M-9 of Roberts *et al.*

6 g. wet weight per liter in the case of carboy-grown cells to 20 g. per liter in the better aerated fermentor, which ultimately replaced the carboy as a culture vessel.

Results

The intercellular space (S_{in}) for *E. coli* with centrifugation for 20 minutes at 20,000 *g*, and with inulin as the non-penetrating solute, was found to be 6.7% ± 1.0 of the total cell pack; probability of this deviation from the mean in a single tube estimation was less than 5%. This S_{in} value was applied as the correction factor in subsequent data unless otherwise stated.

When the citrate space (R) was measured at 0° C. in 0.067 *M* phosphate buffer + 0.9% NaCl at pH 6.4 with a citrate concentration of 2.0 mg./ml. a mean value of 22.8% (standard deviation 1.1) was obtained. These conditions were chosen arbitrarily for reference in subsequent comparisons and apply to subsequent data unless otherwise stated.

Comparison of Citrate with Sulphate Space

Roberts *et al.* (21) have reported that space values for potassium sulphate and a number of other substrates in *E. coli* are constant at about 70% and have suggested that the cell is unable to distinguish between small molecules. The comparatively low values obtained with citrate in the preceding experiment suggested that perhaps there was an exception in the case of the citrate molecule. Accordingly, isotopically labeled potassium sulphate ($K_2S^{35}O_4$) was employed in conjunction with citrate; each substrate was present at the same time, to eliminate unnecessary variation. A comparison of the spaces found for these two compounds is given in Table I, together with the original data for the replications. The results suggest that sulphate and citrate enter identical volumes of the cell. The citrate space was somewhat

TABLE I

SPACE IN *E. coli* AVAILABLE TO SULPHATE AND CITRATE*

Substrate	Weight of cell pack, g. (W_p)	Initial substrate concentration, mg./ml.† (C_0)	Final substrate concentration, mg./ml.† (C_f)	Corrected space, % (R)
Citrate	9.8	0.667	0.530	35.8
Citrate	10.4	0.667	0.528	33.4
Citrate	9.8	0.667	0.524	37.9
Sulphate	9.8	131.5	104.2	35.7
Sulphate	10.4	131.5	103.1	35.3
Sulphate	9.8	131.5	104.2	35.7
Sulphate‡	9.7	131.5	106.8	31.1

* Experimental conditions: 0° C., 0.067 *M* phosphate buffer at pH 6.4. Fifteen milliliters of substrate (V_s) and an intercellular space (S_{in}) of 6.7% apply to all of these data. Citrate and sulphate both present.

† Concentration of sulphate is given as thousands of counts/min. using $K_2S^{35}O_4$. The initial total sulphate concentration was 1 mg./ml.

‡ Determined in the absence of citrate.

higher than that found in the preceding experiment; the reasons for this difference were subsequently investigated in detail. In addition it was found that the space values in both cases were substantially lower than those reported by Roberts *et al.* (21); comparably low sulphate space values were also obtained when $K_2S^{35}O_4$ was used alone in the presence of pyruvate (which is known to be metabolized and thus in itself gives a meaninglessly high space), and when an effort was made to duplicate the procedures described by Roberts *et al.* Experiments to explain this important inconsistency failed to give conclusive information. Mitchell and Moyle (17) similarly were unable to reconcile their findings on phosphate uptake by *E. coli* with those of Roberts *et al.*

The Effect of Energy Sources

The reported ability of *E. coli* to break down citrate in the presence of an energy source such as glucose (8, 15) suggests that glucose may act in allowing the citrate to pass across an otherwise impermeable membrane by some sort of active transport. An experiment undertaken to study this possibility did not show any significant difference in citrate space in the presence or absence of two levels of glucose at any of several temperatures, as shown by the data in Table II. A similar set of experiments in which the exposure time was extended to 30 minutes gave comparable results. The addition of pyruvate also did not affect the citrate space.

TABLE II

THE EFFECT OF AN ENERGY SOURCE ON THE CITRATE SPACE IN *E. coli*

Energy source	Molarity	Temperature, °C.	*R*,* %
Glucose	0.05	0	22.0(21.0)
Glucose	0.005	37	19.0(21.0)
Glucose	0.011	0	18.0(20.0)
Glucose	0.011	34	25.0(22.6)
Glucose	0.011	50	31.0(27.2)
Isocitrate	0.01	34	18.0(22.6)

* Space values in parentheses are control citrate space values determined in the absence of an energy source.

Similarly, we did not detect an additive oxygen uptake in the presence of both citrate and glucose, and the rate returned to the endogenous level after all the glucose was used.

Effect of Time

The citrate space was independent of time, but the lower limit of 10 minutes, which was required for packing, restricted the usefulness of a kinetic analysis with the methods employed.

Absence of Adaptation to Citrate Oxidation

Although neither the presence of an energy source nor short exposure periods affected citrate uptake or oxidation by *E. coli*, there remained the

possibility that either function might, as in other cases, be adaptive either through induced biosynthesis of a necessary enzyme or selection of a mutant. The possibility was examined in manometric and growth experiments.

Extension of the exposure time (8 hours) to citrate in either the presence or absence of low glucose levels did not give the characteristic lag and autocatalytic oxidative response reported for other organisms (2, 9, 14). Moreover, mass inoculation and extended incubation of *E. coli* in Koser citrate broth (with or without added succinate, aspartate, acetate, glutamate, glucose, or peptone at 0.1% concentration) or on Simmons citrate agar failed to alter the oxidative capacity of the organism. Davis (9) also has reported that *E. coli* cannot be induced to utilize exogenous citrate. Szulmajster *et al.* (26), however, were able to induce another strain (M) to utilize citrate by incorporating citrate in the growth medium or by incubating stationary-phase cells for 16 hours with citrate and glucose.

These growth procedures also were used in an unsuccessful attempt to obtain a citrate-oxidizing mutant of this strain of *E. coli*. Turbidity developed in all cultures of the supplemented citrate broth, and a small increase in numbers were presumed to occur even in the absence of supplements (22). The suspensions were streaked on Simmons citrate agar and examined for citrate-oxidizing mutants; none were ever found. In passing, it should be noted that citrate-utilizing mutants of *E. coli* have been reported (19, 29), although only certain strains appear to produce such mutants. At the end of several weeks our cultures were analyzed for citrate content; in the supplemented media, about 20% of the original citrate was found to have disappeared. This finding is in agreement with previous reports (15, 27).

The Effect of Temperature

If the citrate space were dependent on a metabolic pathway, this space should be greatly increased by an increase in temperature in the physiological range. Moreover, it should either decrease abruptly at relatively high temperatures (50° C. or above), at which most enzymes are inactivated, or increase markedly due to the death of the cell and subsequent disruption of the barriers which restrict penetration. On the other hand, if the space being measured is one that depends on diffusion, no abrupt change should occur, although one might expect an upward trend as temperatures increased in the physiological range. Space due to adsorption would tend to decrease as temperature increased. As shown by the solid line in Fig. 1, a gradual upward trend ($Q_{10} = +2.7$ between 0° acid 40° C.) was obtained. Although these data do not necessarily exclude the possibility of an adsorptive mechanism, they are more in agreement with the possibility that the size of the citrate space is dependent on free diffusion, and they apparently negate enzyme involvement. The apparent onset of a rise in the curve at 60° C. also was observed in another experiment (see broken line in Fig. 1) but was about 20° lower; in this second case a space value of 65% was obtained at 50° C. The results at these higher temperatures were variable.

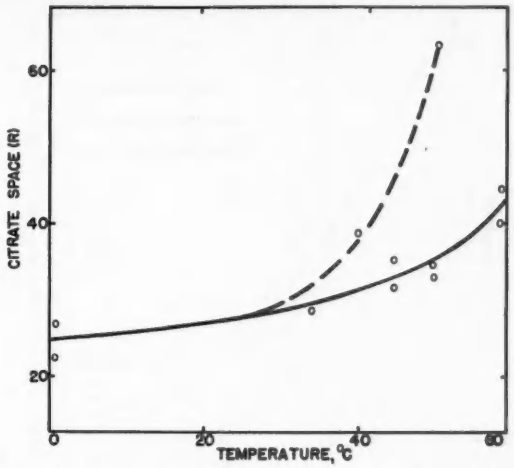

FIG. 1. The effect of temperature on the citrate space of *E. coli*.

Attempts to induce citrate oxidation by heat were only sporadically successful and the results could not be repeated at will. In a few instances brief heating of the cells at 100° C. reduced the endogenous more than the exogenous respiration; representative data are given in Table III. The results are reminiscent of the heat activation of enzymes in other systems (5, 13, 24).

TABLE III

THE EFFECT OF BRIEF HEATING OF *E. coli* ON SUBSEQUENT RESPIRATION IN THE PRESENCE AND ABSENCE OF CITRATE*

Exposure to 100°, sec.	μl. O_2 uptake hour/100 mg. dry weight		
	With citrate	Without citrate	Difference
0	840	850	−10
12	750	530	+220
14	350	110	+240
15	110	50	+60

* Citrate concentration 6.4 mg./ml. in 0.067 *M* phosphate buffer at pH 6.8. Oxygen uptake measured at 34° C.

The Effect of Hydrogen Ion Concentration

Hydrogen concentration often exerts a marked effect on the rate of metabolism of weak organic acids by intact cells, and in some cases this pH response apparently is due to differences in cellular permeability to dissociated or undissociated molecules (10). Moreover, the strong chelating ability of citrate and its possible adsorption by the cell could be expected to vary with pH. Consequently it seemed likely that the citrate space of *E. coli* might be a function of pH. To test this possibility, cells were grown and

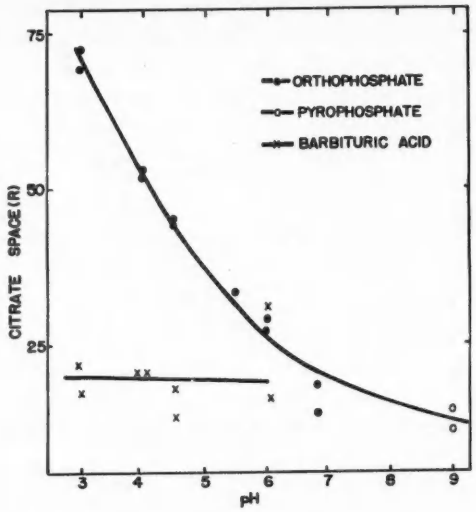

FIG. 2. The effect of pH on the citrate space in *E. coli*. The buffers were used at a concentration of 0.067 *M* and citrate, at 0.2 mg./ml.

prepared in the usual manner but were washed twice with phosphate buffer at the desired pH (pyrophosphate buffer was used for pH 9.0). As shown by the data in Fig. 2, the effect of pH on citrate uptake was marked, the space rising abruptly from about 20% at neutrality to 70% at pH 3.0. If low pH affected the cells themselves the change was not permanent, for on return of the cells to a higher pH there was a concomitant decrease in space. The effect of pH seems to depend in part on the type of buffer used, for when barbituric acid buffer (0.067 *M*) was substituted for phosphate, essentially no change in space was observed over a pH range from 3.0 to 6.0.

Citrate oxidation over a similarly wide range of phosphate-buffered pH, in contrast to the space values, remained relatively constant and indistinguishable from the endogenous respiration.

The Effect of Citrate Concentration

If the space values observed with citrate are due only to adsorption by the cell surfaces, then the space value should decrease with an increase of citrate concentration; a similar relationship at a lower range of concentration would be expected for adsorption on enzyme transport sites. On the other hand, if the externally added solute were equilibrating with the cell water, the space should remain constant with an increase of citrate concentration. A clear decision between these two premises was not obtained from space values alone: the data represented in the left-hand graph of Fig. 3 show that at low levels of citrate the space decreases sharply but then increases until it reaches a plateau. The peculiarly shaped space curve may represent a composite of

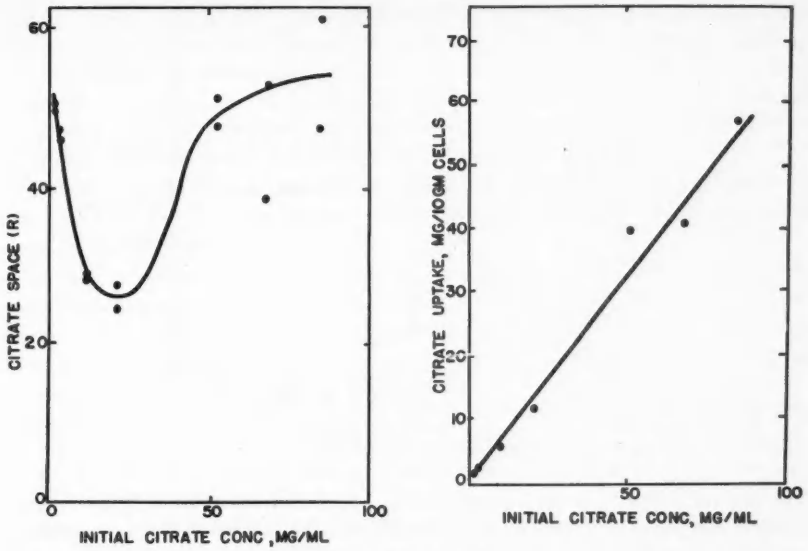

FIG. 3. The effect of citrate concentration on the citrate space (left) and on uptake (right). Both curves are plotted from the same data, obtained at pH 6.0, 0° C., in 0.05 *M* $CaCl_2$. A comparable trend but with smaller values were obtained in the reference experimental conditions.

several different occurrences first, an adsorptive saturation of sites on the cell at very low concentrations of citrate: second, an outflow of water from the cell as a result of increasing osmotic pressure coupled with diffusional entry of citrate into the cell. The important conclusion to be gained from the space data is simply that the citrate space varies considerably with citrate concentration.

When the same data are replotted, however, as in the right-hand graph of Fig. 3, proportionality is found to exist between the citrate concentration in the test solution and the amount lost to the cell pack. Except at very low concentrations, adsorption thus does not appear to be a major factor in citrate uptake by *E. coli* under these conditions.

Attempts to induce citrate oxidation by changing citrate concentration at several pH levels were unsuccessful.

Some Effects of Ion Species and Concentration

The finding that in some cases amino acid penetration and the chelating properties of citrate depend on inorganic ions suggested the desirability of investigating this variable with respect to the citrate space of *E. coli*. In the initial experiments, fermentor-grown cells were washed once with 0.1% gelatin–saline (an unusual and apparently critical step, as it later developed), once in distilled water, and finally in the desired ionic solution. Then the usual space determinations were made with citrate (2 mg./ml.) in the presence of

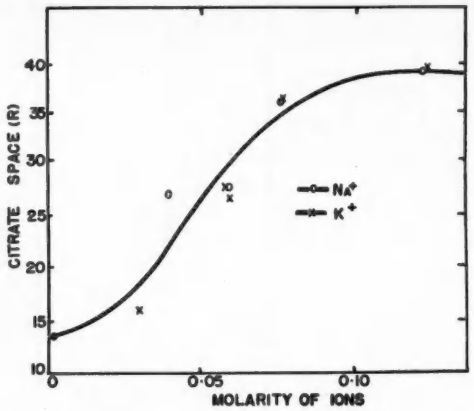

FIG. 4. The effect of monovalent cation concentration on the citrate space of *E. coli* cells prewashed with gelatin–saline.

the ion variables. The data presented in Fig. 4 indicate that varying concentrations of potassium or sodium chloride may influence the citrate space in *E. coli*. A maximal ionic effect was obtained at a molarity of about 0.1 *M* with either salt; in the absence of ions the cells showed a very low space value.

When calcium was used in place of the monovalent cations, the concentration required for the maximal citrate space dropped to about 0.05 *M* (Table IV);

TABLE IV

THE EFFECT OF CATION SPECIES AND CONCENTRATION ON THE CITRATE SPACE IN *E. coli*

Salt	Molarity	pH*	*R*, %
$CaCl_2$	0.25	4.8	30.9
$CaCl_2$	0.050	4.8	39.8
$CaCl_2$	0.100	4.8	41.0
NaCl	0.025	5.0	21.7
NaCl	0.050	4.9	22.3
NaCl	0.100	4.8	24.9
$MgSO_4$	0.012	5.2	23.2
$MgSO_4$	0.025	5.3	18.3
$MgSO_4$	0.050	5.3	20.8
$MgSO_4$	0.100	5.5	26.8
$MgCl_2$	0.012	5.5	15.2
$MgCl_2$	0.025	5.3	20.5
$MgCl_2$	0.050	5.4	24.5
$MgCl_2$	0.100	5.4	29.7
$FeCl_3$	0.006	5.0	80.5
$FeCl_3$	0.012	4.6	535.0
$FeCl_3$	0.025	3.7	—
$FeCl_3$	0.050	2.5	—

* Phosphate buffer was omitted so as to avoid insoluble precipitates; consequently the pH was lower and more variable than usual.

but in terms of normality, and assuming complete dissociation of the calcium chloride, there was no change in optimum from the monovalent system. In this second experiment, however, it was observed that an increase in sodium chloride concentration now only slightly affected the citrate space. The only apparent difference between the two experiments was that the cells used in the first had been washed in gelatin–saline solution.

When magnesium ions were present either as chloride or sulphate, comparatively little change in citrate space occurred (Table IV); the anion apparently is of little consequence.

In the presence of trivalent iron, a dramatic change in citrate space was observed. A very high citrate space (80.5%) occurred with even 0.006 *M* ferric chloride; at higher iron levels, citrate space calculations became meaningless and citrate could not be recovered in the supernatant nor even in washings from the cells in measurable quantities (more than 10% of that lost). The most likely explanation is that the citrate was firmly bound to the cells in the presence of and in relation to the concentration of iron. It seemed unlikely that Fe^{+++} could stimulate metabolism of citrate at 0° C. to a rate that would account for the disappearance of massive amounts of citrate; moreover, even at 37° C. the addition of high levels of Fe^{+++} failed to result in measurable oxidation of citrate.

These cursory experiments on ion effects serve to demonstrate that the citrate space varies with ion concentration and species and that even apparently trivial variations in the procedure can affect the results.

The Effect of Drying, Freezing, and Solvent-treating Cells

Lara and Stokes (12) have shown that dried cells of *E. coli* are able to oxidize citrate at an appreciable rate and our results confirmed their finding. Therefore, it was of interest to determine what effect this drying would have on the citrate space. If the changed oxidative capacity were the result of disruption of permeability barriers, as suggested by Lara and Stokes, then one might expect nearly all the cell water (about 75% of the cell) to be available to citrate and consequently a citrate space of about 75% should be observed. Two experiments to test this prediction were made with cells dried by their procedure (12) and then rehydrated. Both trials give a citrate space (R) of only 47% (Table V); in both, S_{in} values were determined concurrently so as to compensate for possible changes in this correction value. These space values do not represent the degree of increase one would expect for complete permeation, although the cells after drying are now able to oxidize exogenously added citrate.

In contrast to the effects obtained by Foulkes (11) in yeast, freezing the cells did not cause any change in the space available to citrate penetration, nor was the oxidation of citrate affected.

Solvents such as toluene, butanol, and chloroform have been reported to enhance permeability in several instances (11, 16); leakage of large molecular components has been demonstrated in solvent-treated *E. coli* (20). The

TABLE V

THE EFFECT OF VARIOUS TREATMENTS OF *E. coli* ON THE CITRATE SPACE*

Treatment	Temperature, °C.	*R*,† %
Drying	0	47.0
Freezing	0	27.5
Freezing	37	29.8
Toluene	0	30.6
Toluene	37	89.4
Butanol	0	65.3
Butanol	37	250.0
Chloroform	0	43.4
Chloroform	37	46.7

* Experimental conditions: drying done *in vacuo* over P_2O_5; freezing, 10 minutes at −70° C.; solvent treatment, 2 ml. of solvent shaken with 30 ml. of heavy cell suspension (0.5 g. wet wt./ml.) for 5 minutes at 28° C. Cells were then packed for 20 minutes at 20,000 *g* and the solvent saturated supernatant poured off. Cells were resuspended in citrate (2 mg./ml.) and space was determined as previously described with the exception that phosphate was not present.

† S_{in} values used for calculating *R*: dried cells, 17.7%; butanol-treated cells, 13.3%; others not determined, 6.7% used.

effect of solvents on the citrate space and on citrate oxidation in *E. coli* was examined by first shaking the cells in a saturated solution for 5 minutes and then exposing them to 2.0 mg./ml. of citrate at 0° or 37° C. The results are included in Table V. Butanol treatment increased the citrate space at 0° almost to the value predicted for complete permeation and at 37° C. to a level explainable only by adsorption or metabolism. Unexpectedly, toluene treatment resulted in little or no change in the citrate space at 0° C., although the higher temperature was accompanied by a high space value, again suggesting metabolic removal of citrate. Chloroform treatment showed a moderate increase in citrate space which was independent of temperature; however, in no case did solvent treatment of the cells bring about oxidation of citrate. The high space values at 37° C. obtained after butanol or toluene treatment seem best explained as metabolic removal by one or more of the non-oxidative systems known to exist in the organism (8, 23, 28).

Cell extracts prepared by means of sonic oscillation or by treatment with lysozyme and versene showed oxygen uptake in the presence of citrate, confirming previous findings (1, 25).

Citrate Uptake by Cell Walls

Conway and Downey (7) have demonstrated that the cell-wall region of the yeast cell may account for all the uptake of certain solutes and some of the uptake of others. Extending this finding, Britt and Gerhardt (3) have shown not only that the non-pooled lysine uptake in *Micrococcus lysodeikticus* occurs exclusively in the cell wall, but also that the lysine is concentrated there. Consequently, from the beginning of our study we considered it likely that

lower citrate space values represented cell-wall uptake in *E. coli*. A direct test of this concept was made by subjecting masses of *E. coli* to sonic vibration and differentially centrifuging the large particle fraction, which appeared to be made up largely of cell walls. When this preparation was exposed to citrate (2 mg./ml. pH 6.4, 0° C., in water), an R value of 49.0% was obtained. If this same value can be applied to the intact cell and if one assumes that the cell wall of *E. coli* accounts for about 20% of the whole cell volume, then cell-wall uptake in the intact cell would approximate 10% of the whole cell volume, a value lower than the 20 to 30% citrate space usually found. However, the tenuous assumptions of this logic and the possibility of moderate concentration by cell-wall adsorption make it necessary to assume that lower citrate space values may represent only cell-wall uptake.

Discussion

We have seen from the foregoing experiments that the citrate space of *E. coli* is a variable and sometimes capricious measure of citrate uptake, requiring cautious interpretation. The apparent fraction of the cell available to citrate was found to range from about 15% to infinity depending on external variables; the "normal" space was about 25%. The lowest values almost surely represented the uptake of citrate by the cell-wall region, and the "normal" space also could very well be ascribed to such peripheral penetration. Citrate space values extending from about 75%, the probable water content of the cell, to infinity must reflect either non-respiratory metabolism or adsorption of citrate; the latter possibility seems remote, if only from the unreasonably vast number of adsorptive sites required. However, even within the range consistent with the protoplast of the cell, the citrate space varies as the environment is changed. The fraction of the cell that is available to citrate depends on temperature, pH, external citrate concentration, and cation species and concentration; it appears to be independent of an added energy source, time (beyond 20 minutes), and inductional or mutational processes. The changes in space values appear to represent real changes in the portion of *E. coli* that is penetrated by citrate. Unless the changes are peculiar to the citrate molecule, they are counter to the report by Roberts *et al.* (21) that the sulphate space of *E. coli* is independent of temperature, external concentration, and other variables, and to the general inference that such a space value is an independent function. Apparently, variability induced by changes in experimental conditions must be considered when applying this technique for measuring uptake.

A picture of the mechanism of uptake prevailing can be drawn from the effects of several variables on the citrate space. Distinction between an "active" and a "passive" process may be made from the lack of effect when glucose is added as an energy source, from the low Q_{10} that characterizes the response of the citrate space to temperature, and from the proportionality that exists between citrate uptake and a broad range of citrate concentration; thus uptake into the citrate space appears to be a diffusional process. Data

bearing on diffusional entry versus surface adsorption appear to substantiate the former case: both the temperature data and the effect of citrate concentration (except at very low levels) suggest diffusion; moreover, the citrate usually can very easily be washed out of the cells by water. The relatively great changes in citrate space that were observed to accompany the pH and even ion alterations possibly may reflect a Donnan equilibrium; this could explain the difference in pH response observed with phosphate buffer, which probably does not penetrate the cytoplasmic membrane (17) and that with barbiturate, which quite possibly does.

Does surface impermeability account for the inability of intact *E. coli* cells to oxidize exogenous citrate? Apparently not, judging by the data so far obtained. Space values well above that accounted for by cell-wall uptake were found at high temperatures, at low pH, at high external citrate concentration, in the presence of cations such as Ca^{++} and Fe^{+++}, and after solvent treatment of the cells—all without a detectable increase in the oxidation of citrate. It is difficult to rationalize the failure of oxidation to take place under these conditions of apparent citrate permeation, especially in the cases of solvent treatment where other measurements (11, 16, 20) also have shown that such conditions disrupt permeability. It is also noteworthy that dried cells, which are able to oxidize citrate, show some increase in citrate space but less than one would expect for complete permeation. There seems to be little correlation between citrate space and the ability of *E. coli* to oxidize citrate. A further negation of the impermeability concept is implicit in the discovery of several enzyme systems in *E. coli* that are capable of anaerobically degrading the citrate molecule (8, 23, 28); unless all such enzymes are located on the cell "surface" presumably the cytoplasmic membrane, citrate must penetrate the interior in order for them to function. Finally, one may note the negation of the theory of citrate impermeability in yeast by Foulkes (11), who observed that freezing increased the citrate space without increasing the metabolism of citrate unless the frozen cells were treated with chloroform. However, such an analogy between organisms may be unwarranted, and in fact a different response to citrate may be seen in organisms more closely related to *E. coli*. The capacity of intact cells of *Pseudomonas* to oxidize citrate has been clearly shown to be an adaptive process while that of extracts is non-adaptive, a difference ascribed to specific, inducible permeability factors in the intact cell (2, 14). A very similar situation apparently prevails in *Aerobacter* (9). In the absence of comparably specific findings with *E.coli*, Cohen and Monod (6) have extrapolated these findings to the conclusion that "*E. coli* is, for all practical purposes, impermeable to it (citrate)". A conclusion at the opposite extreme was reached by Roberts *et al.* (21): "the protoplasm may be likened to a sponge, the cell membrane to a surrounding hair net unable to exclude the entrance or emergence of small molecules." It appears more reasonable, from the data we have presented, to assume that citrate diffuses into the cell wall of *E. coli*, and under various environmental conditions it may penetrate further into the cell.

If so, then what mechanism controls the citrate oxidizing ability of *E. coli*, which becomes apparent on gross disruption of the cells? The data unfortunately provide no answers and few indications. One may speculate that impermeability at an intracellular locus may prevail, a possibility consistent with the important fact that the tricarboxylic acid cycle is functional in *E. coli* (1, 25). The possibility also must be considered that one or more of the enzymes in the citrate oxidizing system is bound *in situ* in an inactive state—a situation variously called inactivation, masking, the Euler effect, and "cryptic-ity". Several such cases have recently been reviewed by Cohen and Monod (6) and interpreted in terms of stereospecific, inducible transport systems, although they did not include two of the most completely studied cases, J. G. Kaplan's study of the alteration of yeast catalase (13) and heat activation of pyrophosphatase and 5′-nucleotidase in *Proteus* (24). It is not known at present whether these or any of several other speculative mechanisms explain why exogenous citrate is not oxidized by an apparently citrate-permeable cell.

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OXIDATION OF COMPOUNDS IN THE KREBS CYCLE BY PULLULARIA PULLULANS[1]

D. S. CLARK[2] AND R. H. WALLACE

Abstract

Studies with intact cells and cell-free extracts of *Pullularia pullulans* showed that the organism has the necessary enzymes for oxidizing most of the compounds of the Krebs cycle. The rates of oxidation of substrates by intact cells were studied in relation to the age of the cells, the concentration of substrate, the concentration of buffer, and the pH of the reaction medium. Cells grown for 36 hours oxidized a greater number of substrates than did those grown in less or greater intervals. Cells grown for 6 and 18 hours had adaptive enzymes for the oxidation of acetate. The respiratory activity of the 36-hour-old cells was generally greater in 0.0143 *M* concentration of substrate than in lower or higher concentrations; it was not affected by changes in concentration of buffer. With cells at pH 6.8, acetate and ethyl alcohol were oxidized as rapidly as glucose; oxalacetate and pyruvate were oxidized at rates of more than one-half and one-third, respectively, that of glucose; alpha-ketoglutarate, fumarate, *cis*-aconitate, and succinate were slowly oxidized; and citrate, isocitrate, and malate were not oxidized. At pH 3.0, the cells oxidized most of the substrates more rapidly than they did at pH 6.8, but they did not oxidize citrate, *cis*-aconitate, nor isocitrate. The organism oxidized malonate, and its enzymatic activity apparently was not inhibited by that compound.

Cell extracts oxidized alpha-ketoglutarate, citrate, fumarate, malate, and succinate. TPN stimulated the oxidation of citrate and the activity of isocitrate dehydrogenase. DPN stimulated the oxidation of malate. Moreover, the activity of succinate dehydrogenase was inhibited by malonate. The results suggest that the metabolism of *P. pullulans* is by means of the reactions of the Krebs cycle.

Introduction

The yeast-like fungus *Pullularia pullulans* is widely distributed in nature and appears to be economically important with regard to the discoloration of paint and the spoilage of wood products, yet very little has been reported in the literature about the metabolic activities of the organism. Clark and Wallace (1) have shown that a strain of *P. pullullans* has constitutive enzymes for the utilization of several sugars; they also have reported that young cells of the organism oxidize glucose through the intermediates of the pentose phosphate cycle, and that older cells use, in addition, some of the reactions of the Embden–Meyerhof system.

Although further work remained to be done to explain more precisely the reactions by which *P. pullulans* metabolizes carbohydrates, investigations were also needed to determine the ability of the organism to oxidize the compounds that are known to be intermediates in the Krebs cycle. The tricarboxylic acid cycle as proposed by Krebs and his colleagues has received widespread consideration for explaining the enzymatic reactions that bring about the breakdown of acetate to carbon dioxide and water. Although the scheme is

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apparently characteristic of metabolism by animal tissue, its existence in the metabolic activities of microorganisms is still the subject of much investigation and controversy. Various workers have presented evidence to show that some or all of the enzymes required for the reactions of the Krebs cycle exist in some species of bacteria, actinomycetes, yeasts, and molds; none have reported, however, about the presence of those enzymes in cells of *P. pullulans*.

The purpose of this study was to investigate various conditions affecting the oxidative activity of *P. pullulans* on the substrates glucose, pyruvate, acetate, ethyl alcohol, and the various compounds of the Krebs cycle.

I. Experiments with Intact Cells

P. pullulans (ATCC No. 11942) was grown with strong aeration in a medium consisting of ammonium nitrate, 0.1%; casein hydrolyzate, 0.1%; magnesium sulphate, 0.05%; potassium monohydrogen phosphate, 0.1%; yeast extract, 0.2%; and glucose, 1%. At selected times of incubation the cells were centrifuged, washed, aerated, and adjusted to standard density; their respiration, as measured by oxygen uptake, was determined by conventional manometric methods (6). The values for endogenous oxygen consumption were subtracted from the totals of oxygen uptake in all instances.

Effect of Age

Oxygen consumption was measured with cells grown for 22, 36, 60, and 120 hours, respectively. It was found that optimum rates of oxidation occurred with the 36-hour-old cultures. Table I shows that acetate, ethyl alcohol, and glucose were rapidly oxidized by 36-hour-old cells; that oxalacetate and pyruvate were oxidized less rapidly; that alpha-ketoglutarate, *cis*-aconitate,

TABLE I

Effect of age of cells of *P. pullulans* on their oxidation of acetate, ethyl alcohol, glucose, pyruvate, and intermediate compounds of the Krebs cycle

Substrates	μl. of oxygen consumed in 100 minutes			
	22 hr.	36 hr.	60 hr.	120 hr.
Acetate	100	602	502	276
Alpha-ketoglutarate	10	31	78	85
Citrate	0	0	0	0
cis-Aconitate	0	44	0	0
Ethyl alcohol	76	738	262	207
Fumarate	38	36	20	7
Glucose	590	796	399	316
Isocitrate	0	0	0	25
Malate	0	33	0	25
Oxalacetate	80	300	257	232
Pyruvate	77	188	106	57
Succinate	0	47	45	9

Note: All tests were made with resting, glucose-grown cells. Main compartment of each test reaction-vessel contained 3 ml. of cell suspension (dry weight of cells, approximately 15 mg.), and 0.5 ml. of substrate (final concentration, 0.0143 *M*). Center wells of all vessels contained 0.2 ml. of 20% KOH. Control vessels contained all components except substrate. Final concentration of phosphate buffer, 0.1 *M* (pH 6.8). Total volume, 3.7 ml.

fumarate, malate, and succinate were slowly oxidized; and that citrate and isocitrate were not utilized. Table I also shows that 22-hour-old cells oxidized glucose almost as rapidly as did 36-hour-old cells, but that they had comparatively little or no oxygen uptake with the other substrates.

The results show that 36-hour-old cells of *P. pullulans* had a much greater oxygen uptake on a greater number of substrates than did 22-hour-old cells; and that 60- and 120-hour-old cells consumed oxygen at slower rates than did the 36-hour-old cells Apparently 36 hours is closer to the age of optimum physiological activity than is 60 hours. The generally low oxygen uptake by 22-hour-old cells suggests that specific enzymes necessary for the oxidation of the various substrates were either non-existent or were present in very small amounts; on the other hand there is the possibility that the glucose-grown cells were not adapted to other substrates.

In a further experiment the activities of very young cells were studied. The organism was grown in media containing glucose and acetate, respectively. Six transfers were made at successively shorter intervals and the cells developing from the final transfers were grown for 6 and 18 hours. Acetate, ethyl alcohol, glucose, oxalacetate, and pyruvate were the only substrates used for studying the rates of respiration, since these compounds were the main ones oxidized by 22-hour-old cells. Six-hour glucose-grown cells rapidly oxidized but one substrate, namely, glucose. Fig. 1 shows that more than 1000 μl. of oxygen were used in 2 hours with glucose, and that less than 50 μl. were used

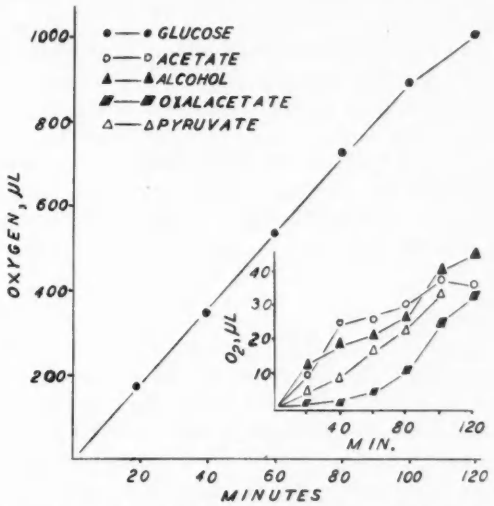

FIG. 1.* Oxidations by 6-hour-old, glucose-grown cells of *P. pullulans*. Dry weight of cells, 20 mg.

*NOTE, FIGS. 1–4. Main compartment of each test reaction-vessel contained 3 ml. of cell suspension and 0.5 ml. of substrate (final concentration, 0.0143 *M*). Center wells of all vessels contained 0.2 ml. of 20% KOH. Control vessels contained all components except substrates. Final concentration of phosphate buffer, 0.1 *M* (the reaction in vessels containing pyruvate, pH 3.0; in all others, pH 6.8). Total volume, 3.7 ml.

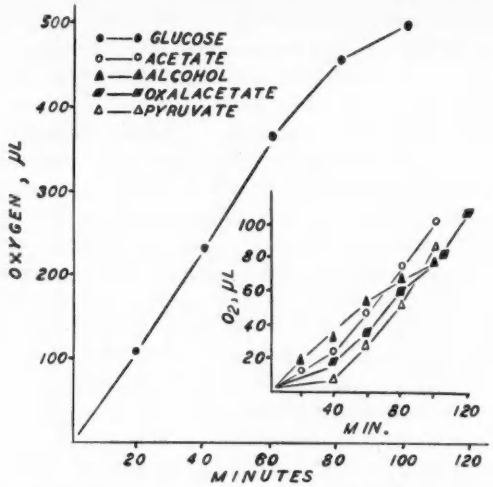

FIG. 2. Oxidations by 18-hour-old, glucose-grown cells of *P. pullulans*. Dry weight of cells, 14.2 mg.

in the same time with each of the other substrates. Similar results are shown in Fig. 2 from an experiment in which 18-hour-old cells were used; it is apparent, however, that these cells consumed approximately twice as much oxygen as did the 6-hour-old cells.

Figs. 3 and 4 show that acetate-grown cells of both ages were capable of rapidly oxidizing acetate and glucose, respectively. The 18-hour-old cells, however, consumed more than twice the amount of oxygen in the presence of ethyl alcohol, pyruvate, or oxalacetate as did the 6-hour-old cells. Results of experiments with 6- and 18-hour-old cells grown in glucose and acetate, respectively, emphasize the suggestion that young glucose-grown cells are deficient in the enzymes required for the reactions of the Krebs cycle. These experiments also showed that the acetate-oxidizing system is adaptive in very young cells.

Concentration of Substrate

P. pullulans was grown in a glucose medium for 36 hours and oxygen consumption was measured of the resulting cells placed in varying concentrations of the respective substrates. Table II shows that as the concentration of most of the substrates was increased from 0.0001 *M* to 0.0143 *M* the amount of consumed oxygen was increased. When the molarity was increased to 0.1429, however, a further increase in oxygen consumption occurred with only four substrates, namely, alpha-ketoglutarate, *cis*-aconitate, oxalacetate, and succinate. Citrate and isocitrate with concentrations above 0.0014 *M* inhibited the endogenous respiration of the cells. Table II also shows that low concentrations of ethyl alcohol were oxidized much more rapidly than were those of glucose. The results suggest that permeability of the cells may be a significant factor regarding the utilization of the various substrates.

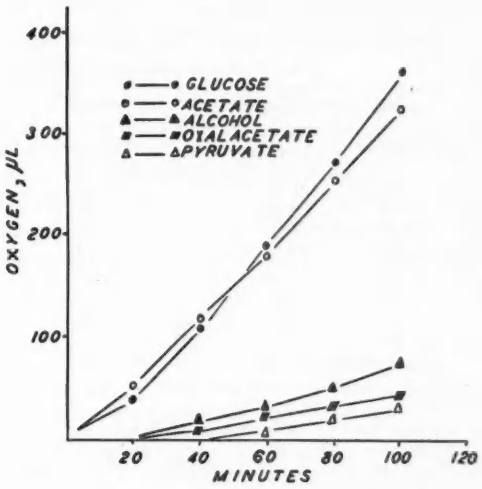

FIG. 3. Oxidations by 6-hour-old, acetate-grown cells of *P. pullulans*. Dry weight of cells, 15.1 mg.

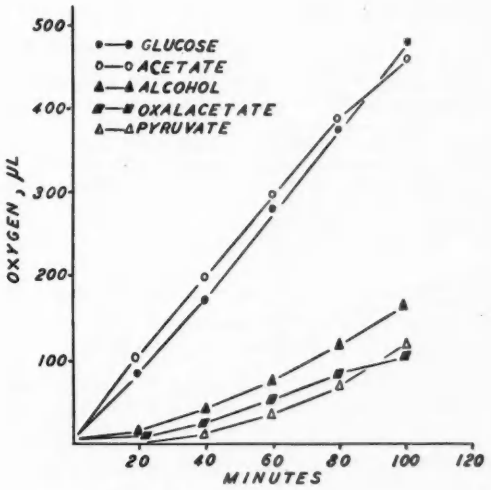

FIG. 4. Oxidations by 18-hour-old, acetate-grown cells of *P. pullulans*. Dry weight of cells, 15.0 mg.

TABLE II

EFFECT OF CONCENTRATION OF SUBSTRATES ON THE UPTAKE OF OXYGEN BY RESTING CELLS OF *P. pullulans*

Substrates	μl. of oxygen consumed in 100 minutes					
	0.0001 *M*	0.0014 *M*	0.0071 *M*	0.0143 *M*	0.0714 *M*	0.1429 *M*
Acetate	0	56	321	589	656	551
Alpha-ketoglutarate	1	6	13	26	61	59
Citrate	0	0	−7	−13	−15	−30
cis-Aconitate	0	−8	9	9	28	29
Ethyl alcohol	2	292	650	707	648	622
Fumarate	0	16	25	29	36	−10
Glucose	6	47	456	715	713	691
Isocitrate	0	6	−5	−15	−50	−46
Malate	0	4	0	7	0	0
Oxalacetate	4	27	106	296	314	338
Pyruvate	7	77	142	186	190	183
Succinate	0	1	7	44	48	92

NOTE: All tests were made with 36-hour-old, glucose-grown cells. Main compartment of each test reaction-vessel contained 3 ml. of cell suspension (dry weight of cells, approximately 15 mg.), and 0.5 ml. of substrate. Center wells of all vessels contained 0.2 ml. of 20% KOH. Control vessels contained all components except substrates. Final concentration of phosphate buffer, 0.1 *M* (pH 6.8). Total volume, 3.7 ml.

TABLE III

EFFECT OF CONCENTRATION OF PHOSPHATE BUFFER ON THE OXIDATION OF SUBSTRATES BY CELLS OF *P. pullulans*

Substrates	μl. of oxygen consumed in 100 minutes		
	0.01 *M* buffer	0.05 *M* buffer	0.1 *M* buffer
Acetate	650	656	589
Alpha-ketoglutarate	60	59	26
Citrate	0	0	0
cis-Aconitate	27	28	9
Alcohol	680	648	707
Fumarate	38	36	29
Glucose	720	713	715
Isocitrate	0	0	0
Malate	1	0	7
Oxalacetate	325	314	196
Pyruvate	200	190	186
Succinate	40	48	44

NOTE: All tests were made with 36-hour-old, glucose-grown cells. Main compartment of each test reaction-vessel contained 3 ml. of cell suspension (dry weight of cells, approximately, 15 mg.), and 0.5 ml. of substrate (final concentration, 0.0143 *M*). Center wells of all vessels contained 0.2 ml. of 20% KOH. Control vessels contained all components except substrates. Final concentration of phosphate buffer, shown in table (pH 6.8 in all instances). Total volume, 3.7 ml.

Concentration of Buffers

P. pullulans was grown for 36 hours in a glucose medium and resuspended in various concentrations of phosphate buffer. Table III shows that the strength of phosphate buffer had little effect on the activity of the cells. The

oxygen consumption of the cells in 0.01 *M* buffer was generally greater than that of cells in the two other concentrations, but the differences in most instances were small. Specifically, however, alpha-ketoglutarate and *cis*-aconitate were oxidized two and three times as rapidly by cells in 0.05 *M* buffer as by those in the 0.1 *M*.

Effect of pH

The results shown in Tables I, II, III, inclusive, indicate that most of the dicarboxylic acids in the Krebs cycle were oxidized very slowly, if at all, by cells in buffer at pH 6.8. Another experiment showed the oxygen consumption of *P. pullulans* at pH 3.0, 6.8, 7.4, and 11.0, respectively. The results given in Table IV show that cells in pH 3.0 buffer oxidized alpha-ketoglutarate,

TABLE IV

EFFECT OF pH OF REACTION MEDIUM ON THE OXIDATION OF SUBSTRATES BY RESTING CELLS OF *P. pullulans*

Substrates	μl. of oxygen consumed in 100 minutes			
	pH 3.0	pH 6.8	pH 7.4	pH 11.0
Acetate	445	637	497	0
Alpha-ketoglutarate	109	36	3	0
Citrate	0	0	0	0
cis-Aconitate	0	6	0	0
Ethyl alcohol	705	789	639	183
Fumarate	269	16	0	0
Glucose	519	808	523	0
Isocitrate	0	0	0	0
Malate	96	0	0	0
Oxalacetate	465	276	50	0
Pyruvate	353	160	65	8
Succinate	161	45	7	0

NOTE: All tests were made with 36-hour-old glucose-grown cells. Main compartment of each test reaction-vessel contained 3 ml. of cell suspension (dry weight of cells, approximately 15 mg.) and 0.5 ml. of substrate (final concentration, 0.0143 *M*). Center wells of all vessels contained 0.2 ml. of 20% KOH. Control vessels contained all components except substrates. Final concentration of phosphate buffer, 0.1 *M* (pH values as indicated). Total volume, 3.7 ml.

fumarate, malate, oxalacetate, pyruvate, and succinate more rapidly than at pH 6.8. At this low pH, the cells could not oxidize the tricarboxylic acids of the Krebs cycle. The cells in pH 7.4 buffer oxidized most of the substrates at slower rates than at pH 6.8. At pH 11.0 only ethyl alcohol and pyruvate were oxidized, the latter only slightly. The final pH of the respective buffers was not altered during the experiment.

The oxidation of an organic acid by *P. pullulans* at a pH less than 3.0 was investigated by using pyruvic acid as the substrate, and 0.1 *M* phosphoric acid at pH 1.4 as the suspending medium. Table V shows that with 0.0143 *M* pyruvic acid the cells consumed 333 μl. of oxygen during 100 minutes, which is only slightly less than the value obtained under similar conditions with cells in pH 3.0 buffer (Table IV). Table V also shows that the organisms consumed

TABLE V

OXIDATION OF PYRUVIC ACID AT pH 1.4 BY RESTING CELLS OF *P. pullulans*

Concentration of pyruvate, *M*	Total μl. of oxygen consumed during times of incubation designated		
	20 min.	60 min.	100 min.
0.0071	28	122	230
0.0143	48	178	333
0.0625	16	52	81
0.1000	−17	−82	−156

NOTE: All tests were done with 36 hour-old, glucose-grown cells. Main compartment of each test reaction-vessel, contained 3 ml. of cell suspension (dry weight of cells, 15 mg.) and 0.5 ml. of solution of pyruvic acid. Center wells of all vessels contained all components except substrate solutions. Final concentration of phosphoric acid, 0.1 *M* (pH 1.4). Total volume, 3.7 ml.

oxygen slowly when the concentration of pyruvic acid was 0.0625 *M*, and that its endogenous respiration was inhibited when the molarity was increased to 0.1. The results of the foregoing experiments showed that pyruvic acid and the dicarboxylic acids of the Krebs cycle are oxidized more rapidly by *P. pullulans* at pH 3.0 than at pH 6.8, which suggests either that the higher pH inactivated the respective oxidizing enzymes, or that the permeability of the cells was greater at the lower pH.

TABLE VI

EFFECT OF CONCENTRATION OF ACETIC, CITRIC, PYRUVIC, AND SUCCINIC ACIDS ON THE UPTAKE OF OXYGEN AT pH 3 BY RESTING CELLS OF *P. pullulans*

Substrates	μl. of oxygen consumed in 100 minutes					
	0.0014 *M*	0.007 *M*	0.014 *M*	0.031 *M*	0.062 *M*	0.125 *M*
Acetic acid	156	500	532	−50	−270	−270
Citric acid	0	0	0	0	−2	−10
Pyruvic acid	60	201	340	350	300	202
Succinic acid	39	70	168	180	209	286

NOTE: All tests were made with 36-hour-old, glucose-grown cells. Main compartment of each test reaction-vessel contained 3 ml. of cell suspension (dry weight of cells, approximately 15 mg.), and 0.5 ml. of substrate. Center wells of all vessels contained 0.2 ml. of 20% KOH. Control vessels contained all components except substrates. Final concentration of phosphate buffer, 0.1 *M* (pH 3.0). Total volume, 3.7 ml.

The results given in Table VI show the effect of the concentration of acetic, citric, pyruvic, and succinic acids on the uptake by cells of *P. pullulans* at pH 3.0. It is noteworthy that acetic acid in concentrations above 0.014 *M* inhibited the endogenous respiration of the cells. High concentrations of succinic acid, however, favored oxygen uptake, the cells consuming almost twice as much oxygen in 100 minutes in 0.125 *M* solution as in 0.014 *M*. Increasing the concentration of pyruvic acid to 0.031 *M* increased cell respiration, but concentrations greater than 0.031 *M* caused a decrease in oxygen uptake.

TABLE VII

LOSS OF RESPIRATORY ACTIVITY OF RESTING CELLS OF *P. pullulans* IN 0.1 *M* PHOSPHATE BUFFER AT pH 3

Substrates	μl. of oxygen consumed in 100 minutes		
	Immersed 1 hr.	Immersed 6 hr.	Immersed 12 hr.
Alpha-ketoglutaric acid	109	22	12
Fumaric acid	269	29	9
Malic acid	96	19	8
Ethyl alcohol	705	50	20

NOTE: All tests were made with 36-hour-old, glucose-grown cells. Main compartment of each test reaction-vessel contained 2 ml. of cell suspension (dry weight of cells, approximately 15 mg.) and 0.5 ml. of substrate (final concentration, 0.0143 *M*). Center wells of all vessels contained 0.2 ml. of 20% KOH. Control vessels contained all components except substrates. Final concentration of phosphate buffer, 0.1 *M* (pH 3). Total volume, 3.7 ml.

The effect of time of exposure of the cells to a suspending buffer at pH 3.0 is shown in Table VII. When the cells were kept for 6 hours at 5° C. in pH 3.0 buffer, they almost completely lost their ability to oxidize various compounds. This finding was significant since in the studies reported here the cells were normally immersed in buffer for about 1 hour before being added to a substrate, which apparently was a sufficient interval to prevent damage or to cause interference with the normal activities of the cells.

Effect of Malonate

Malonate is known to inhibit the oxidation of succinate by yeasts and mammalian muscle tissue (3). The compound is useful, therefore, for determining the possible existence of the Krebs cycle in an organism. If cells of *P. pullulans* are permeable to malonate and they oxidize substrates such as acetate, glucose, or pyruvate through the reactions of the Krebs cycle, their respiration would be inhibited in a mixture of substrate and malonate. Table VIII shows the results of an experiment in which each of the substrates, acetate,

TABLE VIII

EFFECT OF MALONATE ON THE OXIDATION OF ACETATE, GLUCOSE, AND PYRUVATE AT pH 6.8 BY RESTING CELLS OF *P. pullulans*

Substrates	Concentration of malonate							
	0.0000 *M*		0.0143 *M*		0.0625 *M*		0.1000 *M*	
	60 min.	100 min.	60 min.	100 min.	60 min.	100 min.	60 min.	100 min.
Acetate	369	663	356	648	351	648	329	613
Glucose	554	893	553	892	561	904	488	814
Pyruvate	90	195	88	180	75	169	69	102

NOTE: All tests were made with 36-hour-old, glucose-grown cells. Main compartment of each test reaction-vessel contained 2 ml. of cell suspension (dry weight of cells, approximately 17 mg.) 0.5 ml. of substrate (final concentration, 0.0143 *M*), and 1 ml. of a malonate solution. Center wells of all vessels contained 0.2 ml. of 20% KOH. Control vessels contained all components except substrates. Final concentration of phosphate buffer, 0.1 *M* (pH 6.8). Total volume, 3.7 ml.

glucose, and pyruvate, were mixed with various concentrations of malonate. A distinct inhibition did not occur for equimolar concentrations of each substrate and malonate; a slight inhibition of substrate oxidation occurred, however, with 0.1 *M* malonate.

The effect of a low pH on the inhibition of substrate oxidation by malonate was investigated with an experiment in which the cells were suspended in pH 3.0 buffer, and in which the substrates were acetate, pyruvate, and suc-

TABLE IX

EFFECT OF MALONATE ON THE OXIDATION OF ACETIC, PYRUVIC, AND SUCCINIC ACIDS AT pH 3 BY RESTING CELLS OF *P. pullulans*

	Concentration of malonate							
	0.000 *M*		0.0143 *M*		0.0625 *M*		0.1000 *M*	
Substrates	60 min.	100 min.	60 min.	100 min.	60 min.	100 min.	60 min.	100 min.
Acetic acid	422	747	415	694	452	710	432	569
Pyruvic acid	488	776	507	826	422	729	335	630
Succinic acid	170	321	175	326	152	296	120	210

NOTE: All tests were done with 36-hour-old, glucose-grown cells. Main compartment of each test reaction-vessel contained 2 ml. of cell suspension (dry weight of cells, approximately 20 mg.) and 0.5 ml. of substrate (final concentration, 0.0143 *M*) and 1 ml. of a malonic acid solution. Center wells of all vessels contained 0.2 ml. of 20% KOH. Control vessels contained all components except substrates. Final concentration of phosphate buffer, 0.1 *M* (pH 3). Total volume, 3.7 ml.

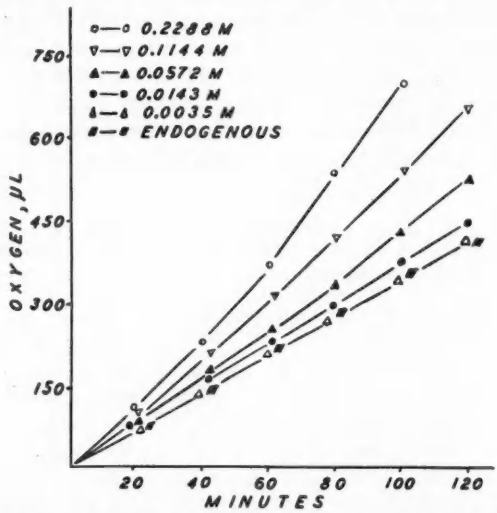

FIG. 5. Oxidation of malonate by 36-hour-old, glucose-grown cells of *P. pullulans*. Dry weight of cells, 15.0 mg. Main compartment of each test reaction-vessel contained 3 ml. of cell suspension and 0.5 ml. of substrate. Center wells of all vessels contained 0.2 ml. of 20% KOH. Control vessels contained all components except substrate. Final concentration of phosphate buffer, 0.1 *M* (pH 3). Total volume, 3.7 ml.

cinate. Table IX shows that 0.1 *M* malonate slightly inhibited the oxidation of the three substrates, but not sufficiently to conclude that interference occurred with the reactions of the Krebs cycle, or specifically with the activity of succinate dehydrogenase.

More attention appears to have been given to malonate as an inhibitor rather than as an intermediate compound in some metabolic process (2). Malonate was used, however, as a substrate to determine if *P. pullulans* is capable of oxidizing that compound. The results given in Fig. 5 show that cells at pH 3.0 have an increased oxygen uptake as the concentration of malonate is increased. This appears to be one of the few occasions in which malonate oxidation by a microorganism has been demonstrated.

II. Experiments with Cell Extracts

P. pullulans was grown with strong aeration in the medium described in Part I. The cells were separated from their growth medium by means of a Sharples centrifuge, resuspended in water, aerated for ½ hour, and collected again in the centrifuge; the cells were again washed twice in this fashion and then stored at 5° C. until required. Cell-free extracts were prepared with powdered alumina in accordance with the method described by McIlwain (4). Conventional manometric methods (6) were used to measure the rates of oxygen consumption by the extracts in various substrates; methylene blue was added to the extracts to serve as a hydrogen carrier.

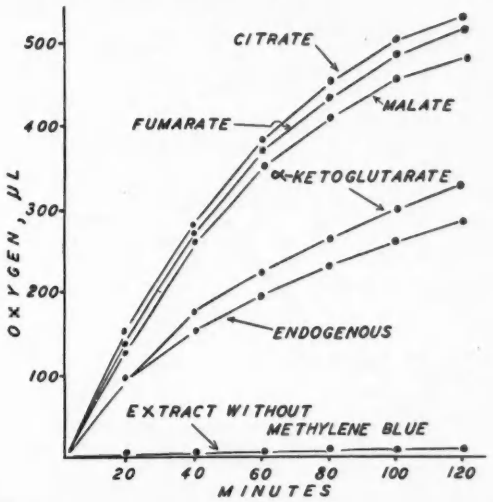

Fig. 6. Oxidation of alpha-ketoglutarate, citrate, fumarate, and malate by a cell-free extract of *P. pullulans*. Extract was prepared from 36-hour-old, glucose-grown cells. Main compartment of each test reaction-vessel contained cell extract, 1 ml. (3.1 mg. of nitrogen/ml.); substrate, 0.5 ml. (final concentration, 0.0143 *M*); methylene blue, 6.5 mg.; $MgCl_2.6H_2O$, 0.23 mg.; and $MnCl_2.4H_2O$, 7.0 μg. Center wells of all vessels contained 0.2 ml. of 20% KOH. Control vessels contained all components except substrates and methylene blue. Final concentration of phosphate buffer, 0.008 *M* (pH 7.4). Total volume, 3.7 ml.

Oxidation of Representative Compounds of the Krebs Cycle

Citrate, alpha-ketoglutarate, succinate, fumarate, and malate were used respectively as substrates for a study of oxygen consumption by cell-extracts. Fig. 6 shows that citrate, fumarate, and malate were rapidly oxidized and that alpha-ketoglutarate was slowly oxidized; succinate was not utilized by the extracts, under the conditions of the experiment. The oxidation of citrate by cell extracts, but not by intact cells, as shown in Part I, suggests that the cell membrane of *P. pullulans* is impermeable to that compound.

Fig. 7 shows the results of an experiment to test the effect of adenosine triphosphate (ATP) and triphosphopyridine nucleotide (TPN) on the oxidation of citrate. The rate of oxidation was increased when ATP and TPN were added separately and together, which suggests that the extract has an active aconitase and a TPN-linked isocitrate dehydrogenase. The presence of the latter was confirmed spectrophotometrically by an experiment showing the reduction of TPN; Fig. 8 shows that a noticeable increase of optical density occurred at a wave length of 340 mμ when TPN was added to a mixture of extract and isocitrate. The results, in conjunction with those in Part I, give good evidence, therefore, that the organism functions in accordance with the reactions of the Krebs cycle.

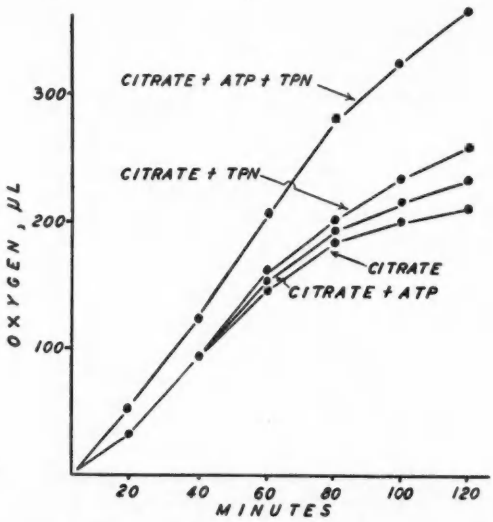

FIG. 7. Effect of ATP and TPN on the oxidation of citrate by a cell-free extract of *P. pullulans*. The extract was prepared from 36-hour-old, glucose-grown cells. Main compartment of each test reaction-vessel contained cell extract, 1 ml. (2.7 mg. of nitrogen/ml.); methylene blue, 6.5 mg.; citrate, 10 mg.; $MgCl_2.6H_2O$, 0.23 mg.; and $MnCl_2.4H_2O$, 7.0 μg. Final concentration of phosphate buffer, 0.008 *M* (pH 7.4). Further additions as shown: ATP, 1.7 mg.; TPN, 0.74 mg. Control vessels contained all components except citrate; citrate, ATP; citrate, TPN; and citrate ATP,TPN. Center wells of all vessels contained 0.2 ml. of 20% KOH. Total volume, 3.7 ml.

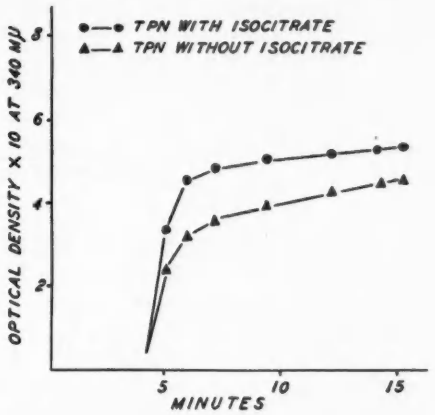

FIG. 8. Evidence for the presence of a TPN-linked isocitrate dehydrogenase in a cell-free extract of *P. pullulans*. Extract was prepared from 36-hour-old, glucose-grown cells. Each test cuvette contained cell extract, 1 ml. (1.3 mg. of nitrogen/ml.); isocitrate, 30 μM.; TPN, 1.2 μM.; $MgCl_2.6H_2O$, 0.23 mg.; and $MnCl_2.4H_2O$, 7.0 μg. TPN was added 4 minutes after start of experiment. Final concentration of phosphate buffer, 0.008 *M* (pH 7.4). Control cuvettes contained all components except isocitrate. Final volume, 3 ml.

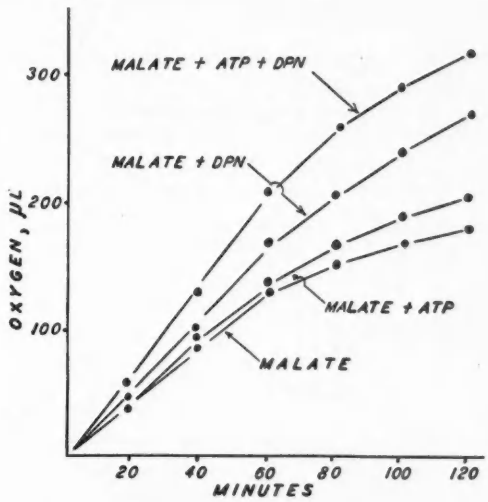

FIG. 9. Effect of ATP and DPN on the oxidation of malate by a cell-free extract of *P. pullulans*. The extract was prepared from 36-hour-old, glucose-grown cells. Main compartment of each test reaction-vessel contained cell extract, 1 ml. (2.7 mg. of nitrogen/ml.); methylene blue, 6.5 mg.; malate, 10 mg.; $MgCl_2.6H_2O$, 0.23 mg.; and $MnCl_2.4H_2O$, 7.0 μg. Final concentration of phosphate buffer, 0.008 *M* (pH 7.4). Further additions as shown: ATP, 1.7 mg.; DPN, 0.74 mg. Control vessels contained all components except malate; malate, ATP; malate, TPN; and malate, ATP,DPN. Center wells of all vessels contained 0.2 ml. of 20% KOH. Total volume, 3.7 ml.

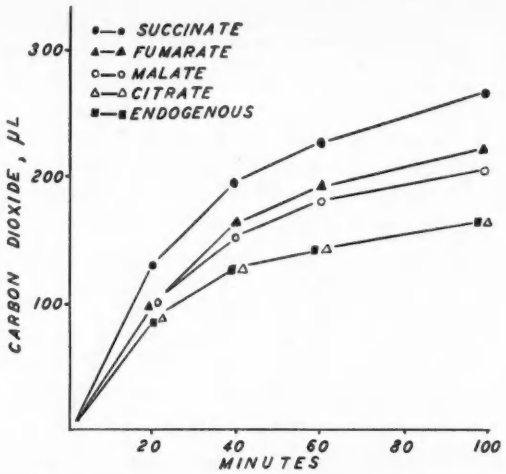

FIG. 10. Evidence for the presence of succinate and malate dehydrogenases in cell-free extracts of *P. pullulans*. The extract was prepared from 36-hour-old, glucose-grown cells. Main compartment of each test reaction-vessel contained cell extract, 1 ml. (3.0 mg. of nitrogen/ml.); substrate, 0.5 ml. (final concentration, 0.0143 *M*); $NaHCO_3$, 2.6 mg.; $K_3Fe(CN)_6$, 2.3 mg.; $MgCl_2.6H_2O$, 0.23 mg.; and $MnCl_2.4H_2O$, 7.0 µg. Control vessels contained all components except substrates. Total volume of each vessel was adjusted to 3.7 ml. with water. An atmosphere of 95% N_2 and 5% CO_2 was used. The pH was 7.0.

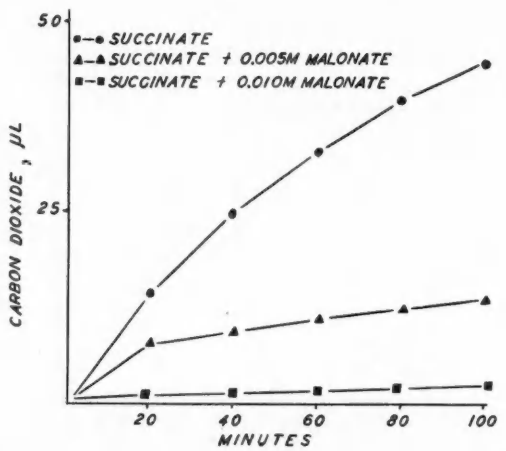

FIG. 11. Effect of malonate on the oxidation of succinate by a cell-free extract of *P. pullulans*. The extract was prepared from 36-hour-old, glucose-grown cells. Main compartments of test reaction-vessels contained cell extract, 1 ml. (1.5 mg. of nitrogen/ml.); substrate, 0.5 ml. (final concentration, 0.0143 *M*); malonate, 0.2 ml. (final concentrations shown on graph); $NaHCO_3$, 2.6 mg.; $K_3Fe(CN)_6$, 2.3 mg.; $MgCl_2.6H_2O$, 0.23 mg.; and $MnCl_2.4H_2O$, 7.0 µg. Control vessels contained all components except substrate. Total volume of each vessel was adjusted to 3.7 ml. with water. An atmosphere of 95% N_2 and 5% CO_2 was used. The pH was 7.0.

Further evidence for the presence of the Krebs cycle may be obtained from a knowledge of the coenzyme linked with malate dehydrogenase. Oxygen consumption was determined of cell extracts in malate mixed with ATP and diphosphopyridine nucleotide (DPN), added singly and together. The results given in Fig. 9 show that the rate of oxidation of malate was increased by DPN, which is evidence that the malate dehydrogenase is DPN-linked in accordance with the Krebs cycle.

Oxidation of Succinate

More extensive studies were required to determine the conditions that would bring about the oxidation of succinate. DPN, ATP, and cytochrome *c* did not stimulate that oxidation. This apparent failure of the extracts to oxidize succinate may have been caused by the inability of methylene blue to act as the primary acceptor of hydrogen from reduced succinate dehydrogenase. In an experiment to demonstrate the presence of succinate dehydrogenase by means of an anaerobic method, as described by Quastel and Wheatly (5), carbon dioxide evolution was measured to give an indirect indication of oxidation. Fig. 10 shows that succinate and malate dehydrogenases were present in cell extracts, and Fig. 11 shows that the activity of the succinate dehydrogenase was indeed inhibited by malonate.

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STUDIES ON THE METABOLISM OF *p*-NITROBENZOIC ACID[1]

NORMAN N. DURHAM

Abstract

Para-nitrobenzoic acid may serve as an oxidizable substrate in addition to other metabolic functions in microorganisms. A strain of *Pseudomonas fluorescens* is capable of using this compound as a sole source of organic carbon and nitrogen for aerobic growth. The enzyme responsible for the oxidation of *p*-nitrobenzoic acid is adaptive in nature. Experimental results with living, irradiated, and dried-cell preparations indicate that *p*-aminobenzoic acid, *p*-hydroxybenzoic acid, and protocatechuic acid are intermediates in the oxidative scheme and the sequence of appearance is in this order. Several other compounds were studied but could not be implicated in the scheme. The nitro group apparently is reduced in vivo to the corresponding amino form. The amino group is then removed as ammonia. By analogy, intermediates in the metabolic scheme of organo nitro compounds might well elucidate the pathway of nitrate reduction.

Introduction

The importance of *p*-nitrobenzoic acid in microbial metabolism was emphasized when Davis and Maas (2) reported that *p*-aminobenzoic acid and *p*-hydroxybenzoic acid were essential metabolites for certain *Escherichia coli* strains, and *p*-nitrobenzoic acid competitively interfered with the utilization of these compounds. A recent report indicates that *p*-aminobenzoic acid may also serve as an oxidizable substrate for *Pseudomonas fluorescens* (3), and subsequent studies have shown that *p*-nitrobenzoic acid apparently does not influence this oxidation (4).

In addition to metabolic studies, the reduction of *p*-nitrobenzoic acid by various systems has also been considered. Bray *et al.* (1) reported that *p*-nitrobenzoic acid was reduced to its corresponding amine in the rabbit intestine and this reduction was bacterial in origin. Saz and Marmur (5) studied the effect of aureomycin on cell-free extracts of *E. coli* (E-26) which reduced the nitro group of *p*-nitrobenzoic acid to the corresponding arylamine. Yamashina (10) reported the preparation of cell-free extracts from *Bacillus pumilus* which reduced aromatic nitro compounds to amino compounds.

These studies offer evidence that *p*-nitrobenzoic acid may be reduced by microorganisms and may also serve as an antimetabolite under certain experimental conditions. However, very little is known in regard to its oxidative metabolism. This paper is concerned with the utilization of *p*-nitrobenzoic acid by *P. fluorescens* and the possible intermediates in the oxidative scheme.

Materials and Methods

The organism used throughout this study was a strain of *P. fluorescens* capable of utilizing *p*-nitrobenzoic acid as a sole source of organic carbon and nitrogen for aerobic growth. Simultaneous adaptation (7) was employed

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as the principal method of investigation. Cell suspensions with the desired enzymatic patterns were produced for manometric experimentation by growing the organism on a synthetic medium containing a single compound as the source of carbon and energy. The synthetic medium used throughout the course of the experimentation was composed of: NaCl 0.2%, KH_2PO_4 0.32%, K_2HPO_4 0.42%, NH_4Cl 0.2%, and agar 2.0%. To this was added 0.1 ml. of a mineral salts solution consisting of $MgSO_4.7H_2O$ 5.0%, $MnSO_4$ 0.1%, $FeCl_3$ 1.0%, and $CaCl_2$ 0.5%. An inorganic nitrogen source, NH_4Cl, was added to the medium in all experiments even though this organism was capable of utilizing *p*-nitrobenzoic acid as a source of carbon and nitrogen. Thus, a medium of constant composition was obtained since NH_4Cl was incorporated into the medium when using non-nitrogenous carbon sources. Experimentation indicated that the presence of NH_4Cl did not significantly influence the results obtained with the organo nitro compounds. The specific compound that served as a source of carbon and energy was added at a final concentration of 0.1 to 0.2%, and the pH was adjusted to 7.2. The "unadapted" cells were grown using asparagine as the carbon source.

All respirometer experiments were performed in the Warburg apparatus (9) at a temperature of 37° C. with air as the gas phase. The enzymatically adapted cell suspensions were prepared by harvesting the growth from plate cultures 20 hours old, washing it twice, and resuspending it in *M*/50 phosphate buffer of pH 7.0. All compounds used as substrates were dissolved in *M*/100 phosphate buffer and adjusted to a pH of 6.8. The substrate concentration was 4 μM. per flask.

Experiments were performed using irradiated cells since ultraviolet light suppresses adaptive enzyme formation. For this work, the cells were placed in a flat bottom Petri plate and exposed to a 15 watt General Electric germicidal lamp for 3 minutes prior to respirometer experimentation.

Dried-cell preparations were prepared by harvesting the growth from plate cultures, washing it twice with phosphate buffer (*M*/50, pH 7.0), and a third time with distilled water. The cell paste was then spread over the bottom of a beaker and slowly dried *in vacuo* at 25° C. The dried residue was ground to a fine powder and stored at 0° C. until used. The dried cells were resuspended in phosphate buffer for manometric experimentation. The oxidation of protocatechuic acid results in the liberation of large amounts of acid; therefore, the phosphate buffer concentration was increased to *M*/10 for both living and dried cells.

Results

Evidence for the Intermediate Roles of p-Aminobenzoic Acid, p-Hydroxybenzoic Acid, and Protocatechuic Acid

The "unadapted" asparagine-grown cells were studied to obtain data concerning the oxidation of *p*-nitrobenzoic acid, *p*-aminobenzoic acid, *p*-hydroxybenzoic acid, and protocatechuic acid by this strain of *P. fluorescens*. The adaptive oxidations of these compounds, as indicated by the marked lags in oxygen uptake after growth on asparagine, are presented in Fig. 1.

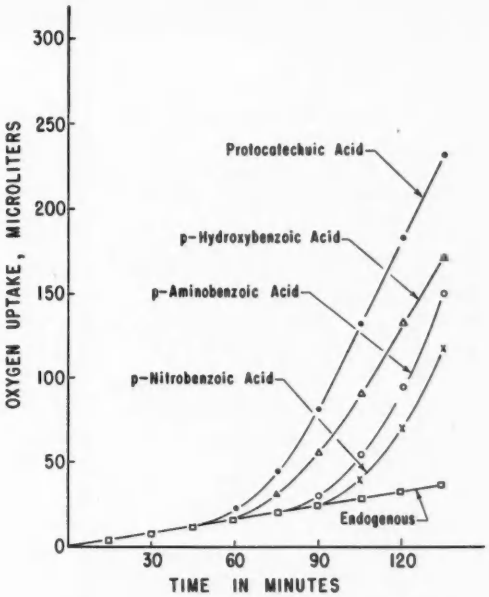


FIG. 1. The oxidation of protocatechuic acid, *p*-hydroxybenzoic acid, *p*-aminobenzoic acid, and *p*-nitrobenzoic acid by *P. fluorescens* grown on asparagine.

Cells grown on *p*-nitrobenzoic acid show simultaneous adaptation to *p*-aminobenzoic acid, *p*-hydroxybenzoic acid, and protocatechuic acid (Fig. 2), which suggests that these compounds are possible intermediates in the oxidation of *p*-nitrobenzoic acid.

The cells grown on *p*-aminobenzoic acid exhibit simultaneous adaptation to *p*-hydroxybenzoic acid and protocatechuic acid, while oxygen consumption on *p*-nitrobenzoic acid was evident only after a marked lag (Fig. 3). These results indicate the *p*-hydroxybenzoic acid and protocatechuic acid appear to be intermediates in the oxidation of *p*-aminobenzoic acid, while *p*-nitrobenzoic acid apparently is not implicated in the scheme.

Additional experiments show that *p*-hydroxybenzoic acid-grown cells are simultaneously adapted to protocatechuic acid, while a period of adaptation was required for the oxidation of *p*-nitrobenzoic acid and *p*-aminobenzoic acid, thus indicating that *p*-nitrobenzoic acid and *p*-aminobenzoic acid are not intermediates in the oxidation of *p*-hydroxybenzoic acid while protocatechuic acid is.

Cells grown on protocatechuic acid exhibit marked lags in oxygen uptake on *p*-nitrobenzoic acid, *p*-aminobenzoic acid, and *p*-hydroxybenzoic acid, suggesting that these compounds are not intermediates in the oxidation of protocatechuic acid.

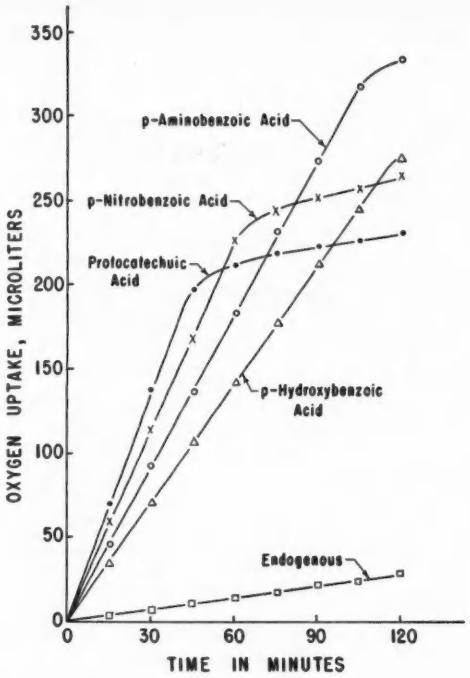


FIG. 2. The oxidation of protocatechuic acid, *p*-hydroxybenzoic acid, *p*-aminobenzoic acid, and *p*-nitrobenzoic acid by *P. fluorescens* grown on *p*-nitrobenzoic acid.

Enzymatic Activity of Irradiated Cell Suspensions

The exposure of cell suspensions to ultraviolet light has been shown to suppress the formation of adaptive enzymes without disturbing the pre-existing enzymatic activities of the cells. The enzymatic activity of irradiated cell suspensions, as measured by oxygen uptake, was studied to determine if ultraviolet light influenced the formation of adaptive enzymes to the substrates in question.

The asparagine-grown cells were irradiated and the results indicated that ultraviolet light suppressed the formation of adaptive enzymes for *p*-nitrobenzoic acid, *p*-aminobenzoic acid, *p*-hydroxybenzoic acid, and protocatechuic acid. Similar results were obtained when catechol and benzoic acid were used as substrates. These data present additional evidence that the compounds in question are truly adaptive substrates. When the cells grown on *p*-nitrobenzoic acid were irradiated, there was no significant difference in the utilization of *p*-aminobenzoic acid, *p*-hydroxybenzoic acid, or protocatechuic acid. A marked difference in the rate of oxygen consumption with irradiated and non-irradiated cells was observed when catechol, anthranilic acid, and

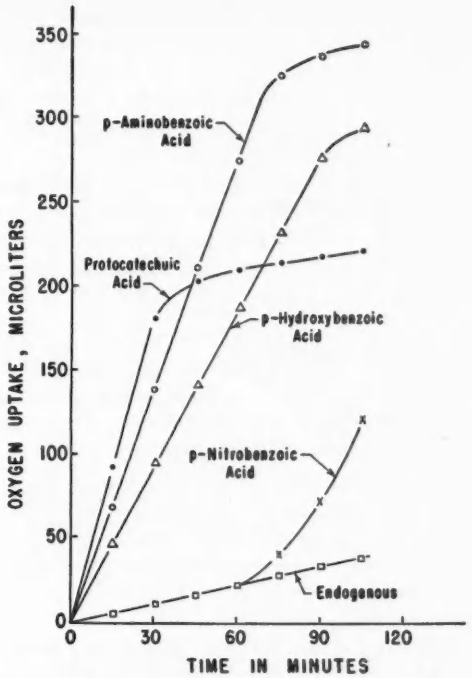


FIG. 3. The oxidation of protocatechuic acid, *p*-hydroxybenzoic acid, *p*-aminobenzoic acid, and *p*-nitrobenzoic acid by *P. fluorescens* grown on *p*-aminobenzoic acid.

benzoic acid were employed as substrates. Irradiation appeared to suppress the enzymes formed in response to these compounds. Fig. 4 shows the typical results obtained with irradiated (U.V.) and non-irradiated cells when *p*-nitrobenzoic acid, *p*-aminobenzoic acid, and benzoic acid were employed as substrates. Since irradiation did not significantly alter the oxidation of *p*-aminobenzoic acid, *p*-hydroxybenzoic acid, and protocatechuic acid, these data offer further evidence that these compounds may be considered to be intermediates in the oxidative pathway of *p*-nitrobenzoic acid.

Enzymatic Activity of Dried-Cell Preparations

Sleeper *et al.* (6) reported that high levels of enzymatic activity for substrates could be found in dried cells when the substrate employed for growth was one known to cause complete adaptation to the compounds in question. Thus, the enzymatic activity of dried cells with pre-established adaptive patterns was studied to obtain additional data. Table I shows the enzymatic activity of the dried cells after growth on the various substrates.

Dried-cell preparations of organisms grown on *p*-nitrobenzoic acid showed enzymatic activity for *p*-aminobenzoic acid, *p*-hydroxybenzoic acid, and protocatechuic acid, thus offering further evidence that these compounds

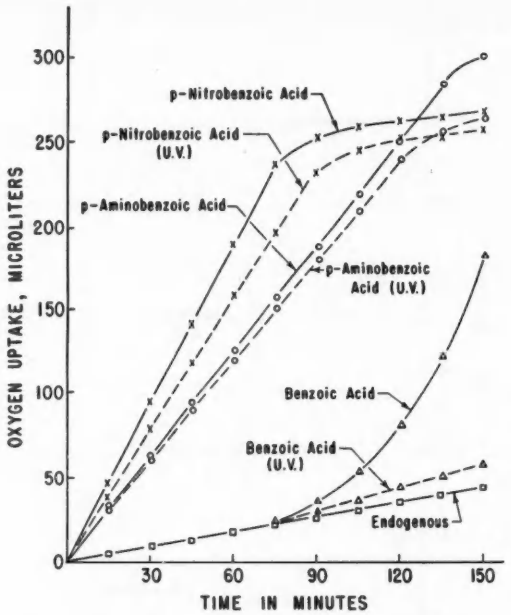


FIG. 4. The oxidation of *p*-nitrobenzoic acid, *p*-aminobenzoic acid, and benzoic acid by irradiated (U.V.) and non-irradiated cells of *P. fluorescens* grown on *p*-nitrobenzoic acid.

TABLE I

ENZYMATIC ACTIVITY OF DRIED CELLS OF *P. fluorescens* ON AROMATIC COMPOUNDS

Cells grown on:	Dried-cell activity						
	p-Nitrobenzoic acid	*p*-Aminobenzoic acid	*p*-Hydroxybenzoic acid	Protocatechuic acid	Catechol	Benzoic acid	Anthranilic acid
p-Nitrobenzoic	+	+	+	+	−	−	−
p-Aminobenzoic acid	−	+	+	+	−	−	−
p-Hydroxybenzoic acid	−	−	+	+	−	−	−
Protocatechuic acid	−	−	−	+	−	−	−
Asparagine	−	−	−	−	−	−	−

are intermediates in the oxidative pathway of *p*-nitrobenzoic acid. Dried *p*-aminobenzoic acid-grown cells demonstrated activity for *p*-hydroxybenzoic acid and protocatechuic acid, but not for *p*-nitrobenzoic acid, while dried cells that were grown on *p*-hydroxybenzoic acid were active for *p*-hydroxybenzoic acid and protocatechuic acid but possessed no activity for other test substrates. Dried protocatechuic acid-grown cells showed activity only for that substrate.

The establishment of adaptive enzyme systems in vivo is essential in order that dried cells may show enzymatic activity for the compounds in question. These results strongly support the data obtained with the living and irradiated cells which suggest that *p*-aminobenzoic acid, *p*-hydroxybenzoic acid, and protocatechuic acid are intermediates in the oxidative metabolism of *p*-nitrobenzoic acid.

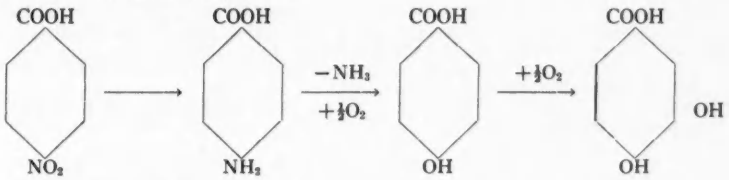
The dried cells oxidized the substrates at a relatively constant rate. However, the total oxygen consumed per mole of substrate was approximately one-fourth the uptake observed with living cells. Extraneous hydrogen carriers, such as methylene blue, did not influence the rate of oxidation of the dried cells.

Oxidation of Additional Aromatic Compounds

In addition to the substrates already studied, the following compounds were tested as possible intermediates: 2,4-dihydroxybenzoic acid, benzoic acid, catechol, *p*-aminophenol, *p*-nitrophenol, *p*-aminosalicylic acid, anthranilic acid, and aniline. Cells grown on *p*-nitrobenzoic acid oxidized benzoic acid, catechol, and anthranilic acid after a period of adaptation, but oxygen uptake in excess of the endogenous respiration was not observed with 2,4-dihydroxybenzoic acid, *p*-aminophenol, *p*-nitrophenol, *p*-aminosalicylic acid, or aniline. Thus, the data indicate that these compounds apparently do not occupy a principal intermediate role in the dissimilation of *p*-nitrobenzoic acid.

Discussion

Experimental results indicate that *p*-nitrobenzoic acid may serve as a sole source of energy for the aerobic growth of a strain of *P. fluorescens*. Thus, this compound fulfills the role of an oxidizable substrate in addition to other metabolic functions in microorganisms. The utilization of *p*-nitrobenzoic acid proceeds via adaptive enzyme formation. This system was studied in regard to elucidating the oxidative pathway. Cells grown on *p*-nitrobenzoic acid are simultaneously adapted to *p*-aminobenzoic acid, *p*-hydroxybenzoic acid, and protocatechuic acid, thus indicating that these compounds are intermediates in the oxidative scheme. Experimentation with irradiated cells and dried cells with pre-established adaptive enzyme patterns strongly support these data. The results indicate that the conclusions may be formulated in the following sequence:



Although the intermediate following protocatechuic acid was not studied in this set of experiments, Stanier *et al.* (8) have reported that protocatechuic acid was oxidized quantitatively to β-ketoadipic acid. Also, the latter part

of the oxidative scheme is in agreement with a previous report (3) in which β-ketoadipic acid was implicated as an intermediate in the oxidation of *p*-aminobenzoic acid.

These results are in agreement with previous reports (1, 5, 10) that *p*-nitrobenzoic acid may be reduced to its corresponding amine by microorganisms, since *p*-aminobenzoic acid was identified as an intermediate in this scheme. Interpretation of the data indicate that the nitro group apparently is reduced to the amino form in vivo, at which time the amino group is then removed as ammonia. Since *P. fluorescens* is capable of utilizing this compound as a sole source of organic carbon and nitrogen, the data obtained from studying the oxidation of this compound might also serve to elucidate the pathway of nitrate reduction. The intermediates immediately following *p*-nitrobenzoic acid in the scheme have not been definitely identified. Experimental evidence indicates that the microbiological oxidation of *p*-nitrobenzoic acid may include *p*-hydroxylamine benzoic acid as an intermediate. By analogy, one might reason that hydroxylamine may also serve as an intermediate in the reduction of nitrate to ammonia.

Additional compounds such as 2,4-dihydroxybenzoic acid, catechol, benzoic acid, *p*-aminophenol, *p*-nitrophenol, *p*-aminosalicylic acid, anthranilic acid, and aniline could be eliminated as possible intermediates by virtue either of their non-utilizability or of their failure to be oxidized immediately by cells adapted to *p*-nitrobenzoic acid.

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LYMPHOCYTIC CHORIOMENINGITIS INFECTION OF MICE AS A MODEL FOR THE STUDY OF LATENT VIRUS INFECTION[1]

J. E. HOTCHIN[2] AND M. CINITS

Abstract

Lymphocytic choriomeningitis virus has been used to set up a model of a latent virus infection in mice. It has been shown to be possible to induce reproducible virus infections in mice which remain completely symptom-free in spite of levels of viral growth equal to those found in the sick animal, by the inoculation of mice within a few hours of birth. This is a convenient method of producing a latent infection in the mice. The effect of the age of the mice at the time of intracerebral inoculation was studied with respect to the pattern of disease produced. Several methods were tried without success in order to induce overt disease in the latently infected animals. The virus did not cause any demonstrable cytopathogenic effect on mouse tissue and several other types of animal tissue. A slight cytopathogenic effect was observed in a strain of human cells in vitro. Virus persisted for weeks in some of the tissue cultures, without damaging the tissue but with the production of active virus. The bearing of the results obtained is discussed in relationship to current concepts of latent virus infection and particularly immunological tolerance. A concept of a special variety of latency is introduced and the name "vital" infection suggested for this.

Introduction

The problems associated with latency in virus disease are commonplace to those closely associated with their investigation, and the subject recurs throughout the literature on viral pathogenesis (1, 19, 29). Different workers have devised various methods of classifying types of latency but, with Andrewes (1), include under this term all those virus infections which do not obviously affect the host. In this paper a latent infection is regarded as one which causes no obvious symptoms nor pathology in the host.

Among the virus diseases of animals there are many examples in which the virus can exist in a latent state; one of the best known of these is lymphocytic choriomeningitis (LCM) of mice. For the reasons outlined below, this virus disease was chosen for study. Initially it was considered that latent LCM infection in mice might prove to be similar to lysogeny in bacteria; however, it appeared that an entirely different process was involved. The results obtained could be best explained by the concept suggested by Burnet and Fenner (9) that under certain conditions a form of immunological tolerance could be exhibited by the host towards the virus. This paper describes the experimental evidence for this conclusion and its relation to the disease of mice caused by this virus.

Lymphocytic choriomeningitis virus was first isolated in 1934 by Armstrong and Lillie (3) from monkeys, and later by Armstrong and Dickens (2) from

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man, by Rivers and Scott (20) from man, and by Traub (30) from mice. At this time the virus was believed to cause latent infection, by Armstrong and Lillie (3) and by Traub (30), who was able to induce signs of the disease in a small proportion of naturally infected mice by the intracerebral injection of sterile broth. Subsequently Traub made extensive observations on the maintenance and spread of LCM in a mouse colony (32, 33, 34, 35, 36). At the start of these experiments some 50% of the mice in the affected colony were found to be naturally infected with LCM without showing the disease. These were immune to intracerebral challenge, whereas in normal mice this caused death with typical convulsions. Some noncarrier mice were immune, but no circulating antibody could be detected by neutralization tests. Traub (33) concluded that mice became immune by "tissue immunity" when they ceased to carry virus after a sublethal infection; during a period of 4 years the virus became only mildly pathogenic for the mice, and ultimately in his colony the virus was transmitted from generation to generation only by intra-uterine passage. In such mice the infection caused only mild signs, and the mice carried the virus for long periods, often many months, in their blood (35, 36). After this close symbiotic relationship had been attained, natural intranasal infection of virus-free mice of that strain with the attenuated virus gave a high proportion of symptom-free LCM carriers, particularly in young mice. The main features of Traub's results were confirmed by Haas (16), who later (17) studied the amounts of virus in different organs of the mouse. A mild infection, leading to a carrier state, resulted from subcutaneous or intranasal inoculation, whereas intracerebral inoculation was always fatal (17). In the mild subcutaneous infections Haas was able to induce lethal convulsions in four out of 24 mice by intracerebral inoculation with cornstarch. This result presumably corresponds with Traub's induction of signs with sterile broth.

The present investigation was conducted with the aim of establishing a convenient, reproducible laboratory model of latency, and its more detailed study. Traub's "carrier state" mice with protracted viraemia were chosen as being highly suitable for this role. Experiments were initiated to determine whether this state could be achieved regularly without relying on gradual symbiosis to occur over a period of years.

Methods

Virus.—Lymphocytic choriomeningitis virus* (W. E. strain, Rockefeller Institute) was obtained in serum from a sick guinea pig, 5 days after infection. It was subsequently passaged twice in mouse brain. All experiments were made with pooled mouse brain (10% emulsion in phosphate buffered saline) from these passages, which was stored in sealed ampoules, immersed in a dry-ice alcohol bath.

*Kindly supplied by Dr. F. P. Nagler, Chief, Virus Laboratories, Laboratory of Hygiene, Department of National Health and Welfare, Ottawa.

Mice.—Swiss albino mice (University of British Columbia strain) were used throughout, except where specified.

Inoculations.—Intracerebral inoculations were made under ether anaesthesia, with 0.02 to 0.03 ml. inoculum. For all other inoculations, 0.03 to 0.04 ml. was used.

Dilutions.—Titrations were made with 10-fold serial dilutions in phosphate buffered saline.

Earle's saline (15).—This was used as a balanced salt solution.

Calcium-free Earle's saline.—This consisted of Earle's saline in which the calcium and magnesium ions were replaced by sodium.

Medium 192/2 (37).—This is a synthetic medium containing balanced salts, vitamins, amino acids, etc., and 0.05 g.% purified bovine serum albumin. It was stored at 4° C. and received 100 units penicillin and 100 μg. streptomycin per ml. before use.

Trypsin solution.—This was prepared as a 5% solution of trypsin 1:300 (Nutritional Biochemicals) in 0.85% saline. It was centrifuged and Seitz-filtered immediately after preparation and was stored at −20° C .

Lactalbumin hydrolyzate.—This was prepared as a 5% solution in 0.85% saline; it was sterilized by autoclaving and stored at −20° C.

Phosphate buffered saline.—This was the same as that used by Dulbecco and Vogt (13).

Calf serum.—Calf blood (obtained fresh at an abattoir) was defibrinated by whipping and the serum centrifuged and stored in bulk at −20° C. It was thawed and Seitz-filtered in small batches as required; after use it was refrozen and stored at −20° C.

Novikoff tumor tissue.—This was obtained from Dr. M. D. Darrach* as a growing intraperitoneal tumor in a Wistar rat.

Cell counts.—These were performed by standard techniques, with a haemocytometer counting chamber.

Tubes.—Tubes routinely used for cultures were Pyrex, size 130×15 mm.; these were stoppered with black rubber stoppers and incubated at an angle of 5° from the horizontal, with the stopper uppermost.

Tissue cultures.—With whole organs, suspensions were made of animal tissues by mincing with scissors or expulsion through a syringe, followed by trypsinization in 0.25% trypsin in calcium-free Earle's saline. Trypsinization was discontinued after 10–20 minutes by cooling and addition of 5% calf serum, and was followed by centrifugation at 200 r.p.m. for 30 minutes or 1000 r.p.m. for 2 minutes, according to the stability of the cell type. The pellet was resuspended in medium and a count was made. When pure cell-line cultures were used, these were grown on glass, and 0.1% trypsin in calcium-free Earle's saline was used to remove and suspend the cells; in other respects the cultures were made as described above.

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Results

Experiments to Establish the Pattern of Infection with the Virus and Host Strains in Use

Stock virus was titrated in 1- to 2-month-old mice by intracerebral and intraperitoneal routes. Results of representative experiments are summarized in Table I. It is evident that after intracerebral inoculation all animals showing signs ultimately died. They exhibited jumpiness, blepharitis, and spontaneous or spinning-induced convulsions, which followed the typical pattern with extended hind limbs. Intraperitoneal inoculation was never fatal; signs consisted of sluggishness, diarrhea, blepharitis, loss of weight, and a huddled posture during the 6th to 12th days after inoculation. A similar experiment with older (3–6 months) mice gave the same results, except that signs appeared slightly earlier (*ca.* 12 hours). Throughout the experimental period the titer of the stock virus remained slightly above 10^3 ID_{50}/ml. Freshly prepared 10% brain suspensions always gave this titer in these mice.

TABLE I

The result of intracerebral and intraperitoneal inoculation of stock LCM virus into groups of four weanling mice

Weanling mice v. dil.	Days after inoculation									Deaths at 20 days
	5	6	7	8	9	10	11	12	14	
Intracerebral inoculation										
Control										0/4
10^{-6}										0/4
10^{-5}										0/4
10^{-4}				S	S	D				1/4
10^{-3}				SSSS	SSDD	DD				4/4
10^{-2}		SSSS	SSSS	DDDD						4/4
10^{-1}	SSSS	SSSS	SSSS	DDDD						4/4
Intraperitoneal inoculation										
Control										0/4
10^{-6}		D								1/4
10^{-5}										0/4
10^{-4}										0/4
10^{-3}		SS	SS	SSSS	SSSS	SSSS	SSSS	SSRR	RR	0/4
10^{-2}		SSSS	SSSS	SSSS	SSSS	SSSS	SSSS	SSRR	RR	0/4
10^{-1}		SSSS	SSSS	SSSS	SSSS	SSSS	RRRR			0/4

Note: S = sickness of mice; D = death; R = recovery.

The survivors of the above experiment were rechallenged by intracerebral inoculation of a 10^{-3} dilution of stock virus. The results are shown in Table II. None of the survivors of the initial intracerebral inoculations survived rechallenge, i.e., none was immune to this inoculum, whereas the initially intraperitoneally inoculated group showed two out of four immune mice in the 10^{-4} and 10^{-5} virus groups, and one out of three surviving in the 10^{-6} virus group; apparently the immunizing titer of the stock virus was considerably higher than the infective titer.

The Effect of X-Irradiation and Cortisone upon LCM Infection in Adult Mice

Rowe (22) reported the ability of a dose of 300 r. of X-rays given prior to intracerebral inoculation of LCM in mice, to protect them against convulsions

TABLE II

THE EFFECT OF INTRACEREBRAL RECHALLENGE, WITH 10^{-3} STOCK VIRUS, UPON THE SURVIVORS OF EXPERIMENT I 4 WEEKS AFTER THE ORIGINAL CHALLENGE

Previous v. dil.	IC challenge v. dil.	Mice at start	Days after IC challenge 8	9	10	11	12	Surviving mice
			Intracerebral					
Control	10^{-3}	4	SSSS	SSDD	DD			0
10^{-6}	10^{-3}	4	SSSS	SDDD	D			0
10^{-5}	10^{-3}	4	SSSS	SSDD	SS	DD		0
10^{-4}	10^{-3}	3	SSS	DDD				0
			Intraperitoneal					
Control	10^{-3}	4	SSSS	SDDD	D			0
10^{-6}	10^{-3}	3	SSS	SSS	DDS	S	R	1/3
10^{-5}	10^{-3}	4	SSSS	DSSS	SSS	SSD	RR	2/4
10^{-4}	10^{-3}	4	SS	SS	SS	DD		2/4
10^{-3}	10^{-3}	3						3/3
10^{-2}	10^{-3}	4						4/4
10^{-1}	10^{-3}	4						4/4

NOTE: S = symptoms; D = death; R = recovery.

and death due to the virus. This result has been confirmed, and a similar effect has been noticed following injections of cortisone acetate in place of X-irradiation. The effect of X-irradiation and cortisone upon the outcome of LCM infection in adult mice is shown in Table III. The X-ray dose given prior to infection furnished considerable protection against the acute cerebral form of the disease, protecting mice for as long as 63 days. After irradiation and intracerebral inoculation with 10^{-2} stock virus, the mice showed slight general signs of sickness after 7 to 10 days; this lasted for about 10 days, after which time the mice appeared perfectly well. However, they slowly became progressively less active and weaker, and were all dead by 9 weeks, whereas irradiated but uninoculated mice were perfectly well at this time.

TABLE III

THE EFFECT OF X-RAYS AND CORTISONE UPON TIME OF DEATH AFTER LCM INFECTION IN MICE

Group	10^{-3} LCM route	Day of cortisone or irradiation relative to challenge	Cortisone or X-ray dose	Average day of death after challenge	Proportion of mice dead by 30 days
1	IC	−1	300 r.	25*	4/8
2	—	−1	300 r.	>60	1/4
3	IC	Nil	Nil	7.5	4/4
4	IC	+6	300 r.	13.3	3/3
5	IP†	+5	300 r.	11.0	2/3
6	IC	+3	300 r.	12.3	3/3
7	IC	+1	300 r.	11.0	
8	IC	Every 3 days after inoc.	1 mg. SC	9.7	7/7
9	Nil	"	1 mg. SC	—	0/8
10	IC	"	2 mg. SC	17	8/8
11	Nil	"	2 mg. SC	18.25	8/8
12	IC	"	3 mg. SC	13	6/6
13	Nil	"	3 mg. SC	18	6/6
14	IC	Nil	Nil	8.2	8/8

NOTE: IC = intracerebral; IP = intraperitoneal.
*This figure represents only half the mice, the remainder being alive at 60 days.
†This group was accidentally inoculated intraperitoneally instead of intracerebrally

When intracerebral inoculation preceded irradiation, partial protection was achieved, but this decreased as the interval between inoculation and irradiation increased. When intraperitoneal inoculation preceded X-irradiation, cerebral symptoms were induced by the irradiation and the mice died in convulsions. This surprising result was observed in a group of mice accidentally inoculated intraperitoneally, and was confirmed by more detailed study.

Cortisone acetate, in a dose of 2 mg. subcutaneously every 3 days, prolonged survival of intracerebrally inoculated mice for 18 days, but control-uninoculated mice given this dosage of cortisone also died at this time.

The Effect of Host Age upon the Outcome of Intracerebral Infection with LCM

This effect was investigated by inoculating a 10^{-2} dilution of virus intracerebrally into groups of 15 mice of different ages. Results are shown in Fig. 1. It can be seen that the number of surviving mice decreases as age increases, and also in older mice the average time of death is earlier than in young mice. The curve relating time of death to age at inoculation can be seen to undergo marked inflection between 4 and 6 days' inoculation time after which it remains relatively constant.

Examination of mice infected intracerebrally shortly after birth revealed that some of these, while having varying degrees of chronic sickness, contained fully infective virus for long periods. This survival in a carrier state was most marked in those mice which had been infected earliest after birth. To investigate this further, four litters of newborn mice (a few hours old) were inoculated intracerebrally with 10^{-1} stock virus. No mice developed any obvious signs. At 8 days, two mice were removed and killed; a 10%

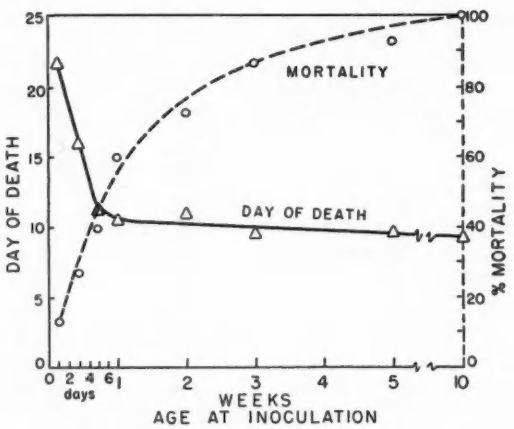

Fig. 1. The effect of age at inoculation upon mortality and time of death after intracerebral infection of mice with LCM virus.

suspension of their brains killed all mice when inoculated intracerebrally into weanlings or adults. At 6 weeks the mice were weaned, and the mothers (none of which had shown signs) were challenged with 10^{-3} stock virus intracerebrally; three out of four were immune; the fourth died with typical LCM. At 8 weeks, two more mice of the experimental litters were sacrificed, their brains pooled in a 10% suspension and titrated by intracerebral inoculation in weanling mice. Results are shown in Table IV. These mice contained the same titer of virus in their brains as that found in the brains of adult mice suffering from a fatal infection after intracerebral inoculation. At this time the mice showed few or no signs, although they grew slightly less rapidly than normal controls. One or two exhibited a dainty "bird-like" behavior and were unusually "jumpy". A larger number of newborn mice were then infected intracerebrally with 10^{-2} stock virus. When larger numbers were required, it was necessary to accept a wider spread of age owing to difficulties in the mouse supply; thus not more than one or two families could usually be inoculated at any one time after birth. When mice were inoculated intracerebrally or intraperitoneally within a few hours of birth, little or no sign of infection was observed during the following 6 months, although virus remained at maximum titer throughout this period in blood, spleen, liver, and brain. However, when mice 1 to 3 days old were used, some of these died during the following 6 weeks and the remainder showed varying degrees of retarded growth.

TABLE IV

TITRATION OF POOLED 10% MOUSE-BRAIN SUSPENSION FROM TWO MICE 8 WEEKS AFTER INTRACEREBRAL INOCULATION WITH 10^{-1} LCM VIRUS WHEN A FEW HOURS OLD. CONTROL BRAIN SUSPENSION CAME FROM AN UNINOCULATED MOUSE OF THE SAME AGE

		Mortality
Control brain suspension (10^{-1})		0/4
Inoculated mice brain suspension dil.	10^{-1}	4/4
	10^{-2}	4/4
	10^{-3}	4/4
	10^{-4}	0/4

EXPERIMENTS USING NEONATALLY LCM-INFECTED MICE

When it had been established that neonatal inoculation (within a few hours of birth) of mice with LCM virus produced a prolonged latent infection in a reproducible way, such latently infected animals were used after weaning for the following experiments.

The Effect of Intracerebral Inoculation of Sterile Material in LCM-infected Mice

Latently infected mice of different ages, produced by neonatal intracerebral inoculation, were given one or more intracerebral injections of sterile broth, starch solution, or milk. At no time did any of these respond by showing signs of LCM; apart from side effects of the inoculation, the mice were

unchanged and their latent infection was unaffected. Intracerebral inoculations of sterile material were also made in mice which had received LCM virus a few days previously, by intranasal, intraperitoneal, or subcutaneous routes. Of these, two out of three of the intraperitoneal group showed cerebral signs 4 or 5 days after they were given sterile broth intracerebrally. Further experiments showed that this effect was observed only when the intracerebral broth inoculation was made between 2 and 6 days after the intraperitoneal virus inoculation. Results in the other groups were indeterminate (a few mice showing slight signs); however, the numbers used were not large (six mice for each group).

It was concluded from these experiments that the intracerebral inoculation of broth does not alter the latent state of LCM infection in neonatally infected mice. However, intracerebral inoculation of broth can induce overt cerebral symptoms if made at a suitable time interval after intraperitoneal inoculation.

The Effect of Cold Stress on Neonatally LCM-infected Mice

Attempts were made to induce signs by cold stress. The following groups of mice were used: normal 6-week-old mice, 6-week-old mice previously infected intracerebrally at birth, and adult mice previously infected intracerebrally at birth. These were maintained for 10 days in a 4° C. cold room. No signs of LCM were induced in these mice.

The Effect of X-Irradiation upon Neonatally LCM-infected Mice

A 300 r. dose of total body X-irradiation did not cause the onset of any signs of LCM in mice of different ages (6 weeks to 6 months) which had been infected with LCM intracerebrally at birth. However, a few mice in the 6-week-old group which already showed minimal signs, e.g., slight weight loss and irritability, were temporarily improved for about 2 weeks by the irradiation. No difference was found afterwards between the brain virus titer of irradiated mice and that of similarly infected controls. However, attention is drawn to the effect of X-irradiation upon intraperitoneally inoculated mice reported below.

The Effect of Viral Superinfection upon Neonatally LCM-infected Mice

Both normal and neonatally LCM-infected mice, aged 2–3 months, were tested in groups of three for their reaction to superinfection with ectromelia

TABLE V

THE EFFECT OF INFECTION WITH WESTERN EQUINE ENCEPHALOMYELITIS AND ECTROMELIA VIRUSES UPON MICE WITH AND WITHOUT NEONATAL LCM

Challenge virus	Intracerebral inoculation at birth	Days after challenge						
		2	3	4	5	6	7	8
WEE (intracerebral)	LCM	—	—	—	—	—	—	—
WEE (intracerebral)	Nil	SSS	SSD	DD				
EM (footpad)	LCM	—	—	—	—	SSS	DSS	
EM (footpad)	Nil	—	—	—	—	DSS	SS	DS

NOTE: Each group contained three mice. S = sick mouse; D = dead mouse.

and western equine encephalomyelitis (WEE) viruses. Results are shown in Table V. It was found that neonatal latent infection with LCM apparently protects against signs or death from WEE, but not against ectromelia. No effect upon the LCM infection by the superinfecting virus was noticed; no symptoms due to LCM were seen. This effect of latent LCM on WEE infection is being more fully investigated.

Tissue Culture Experiments

Various tissues were used in attempts to cultivate LCM, and to obtain a clear-cut cytopathogenic effect so that a plaque-count assay could be used for this virus and for cells carrying it. The following tissues were tested, by established techniques, for their ability to exhibit cytopathogenicity with LCM:

Mouse tissue: kidney, liver, lung, brain, spleen (adult and embryonic tissue was tried in each case), mixed embryonic tissue.

Guinea pig tissue: embryonic kidney, liver, brain, and lung.

Rat tissue: mixed embryo, Novikoff hepatoma (18).

Chick embryo mixed cells.

Human cells: HeLa cells (24), Chang conjunctiva cells (11), oral carcinoma, strain KB (14), strain HS.*

With one exception, none of the above cell types showed cytopathogenicity beyond the first passage, with high titer virus. The exception was the KB cells, which gave a cytopathogenic effect after 8 days, which was maximal by 10 days with a 10^{-2} dilution of stock virus. This effect could be readily obtained in tubes, with 192/2 medium. The cytopathogenic effect was never complete; only about 70% of the cells were affected (rounded, partly detached), and attempts to produce LCM plaques on these cells failed, although plaque assays were readily obtained with herpes simplex, Newcastle disease, and WEE viruses. No cytopathogenic effect was produced in the presence of 5% normal calf serum. Previous sublethal damage to chick cells by ultraviolet irradiation did not render them more susceptible to LCM virus.

Persistence of Virus in Tissue Culture

Several of the above tissues were found to support LCM virus (presumably by growth) over a period of days or weeks. These included mixed chick tissue, mouse brain, and KB cells grown in 192/2 medium.

In spite of viral growth no cytopathogenicity could be detected, although the cultures were kept for 3 weeks; at the end of this period the fluid from the chick tissue and KB cells still caused typical LCM after intracerebral inoculation in mice, but fluid from mouse brain did not. However, the infected chick tissue cultures appeared to be less metabolically active than the controls since it was noticed that they took longer to become acid than the uninfected controls.

*Kindly supplied by Dr. Alice Moore, Sloan-Kettering Institute for Cancer Research, 410 East 68th Street, New York 21, New York.

Egg-adapted Virus in Tissue Culture

In view of the very slight cytopathogenicity of LCM in chick embryo tissue cultures, egg-adapted LCM virus was tested for its possible ability to cause a greater effect. Such a strain of LCM, No. 4707, was kindly provided by Miss Elinor Whitney.* This virus had been through 36 chorioallantoic membrane and 43 yolk-sac passages, but as regards cytopathogenic effect it was found to behave in chick tissue culture no differently from the non-egg-adapted strain.

Interference Experiments in Tissue Cultures Infected with LCM

Since LCM virus caused too weak a cytopathogenic effect for a precise in vitro assay procedure, experiments were made to see if this virus could interfere with other cytopathogenic viruses. WEE virus in chick tissue was first used; 0.1 ml. of stock LCM virus, diluted 10^{-1} or 10^{-2}, introduced into cultures 3 days before 0.1 ml. of high titer WEE virus, was found to prolong the life of the culture for 1 to 3 days, over the time (1 day) during which control cultures were destroyed by WEE. However, this effect was not sufficiently pronounced to enable it to form the basis of an assay procedure for LCM virus. Dalldorf (12) reported a sparing effect of LCM on experimental poliomyelitis (Lansing strain) in monkeys. Consequently the ability of LCM virus to protect HeLa cells from the cytopathogenic effect of poliomyelitis virus was tested; however, no evidence of protection of cultures by LCM against poliomyelitis (Brunhilde strain) was observed in vitro, as judged by the time of onset of cytopathogenic effect due to poliomyelitis virus in normal and LCM-infected cultures.

Conclusions

The Bearing of These Results upon the Concept of Latent Virus Infection

From the preceding experiments the following conclusions were drawn:

1. A reproducible state of latent infection with LCM can be induced by neonatal intracerebral inoculation of mice.

2. This state persists unchanged for at least 6 months.

3. The induction of overt disease due to the latent neonatal infection was not achieved by using cerebral trauma, stress, X-irradiation, or viral superinfection.

4. This type of latent infection with LCM conferred protection against intracerebral infection with WEE but not to footpad infection with ectromelia.

5. Agents (cortisone and X-irradiation) known to interfere with the immune response are able to exert a protective effect upon mice against the symptoms and lethal effects due to LCM infection.

*Division of Laboratories and Research, New York State Department of Health, Albany, New York.

6. No cytopathogenic effect was detected in mouse tissue cultures, and only slight effects were noted in cultures of other cell types after infection with LCM virus.

The properties of the model (intracerebral neonatally infected mice) described in this paper present features which indicate a special category for the type of latency produced. These properties are summarized below with the corresponding properties of the infection in adult mice for comparison.

PROPERTIES OF MICE INFECTED WITH LCM

Infected neonatally	Infected after 1 week of age
1. Carry virus indefinitely	1. If not lethal, virus is ultimately suppressed
2. No neutralizing antibody detected	2. Some neutralizing antibody
3. No cytopathogenicity in vitro. No inflammation in vivo	3. No cytopathogenicity in vitro. Inflammatory response in vivo
4. Same titer of virus as in pathologically infected older mice	4. Same titer of virus as in neonatally infected mice
5. Disease cannot be induced in these mice	5. Disease can be largely suppressed by X-rays or cortisone

These properties indicate that the LCM virus is only harmful to the mouse if the mouse responds to it immunologically as a foreign antigen. If the immune response is inhibited by X-rays or cortisone, the lethal effects, signs, and gross pathological changes of the disease are all inhibited but the virus titer is unchanged. Similarly if infection occurs pre- or neo-natally, the mouse develops immunological tolerance to the virus in a similar manner to that described by Billingham *et al.* regarding homograft tolerance in mice. The parallel relationship between LCM virus and homograft tolerance in mice as shown by these and other workers' results is summarized below.

FACTORS RELATIVE TO THE DEVELOPMENT OF IMMUNOLOGICAL TOLERANCE TO LCM VIRUS AND MOUSE SKIN GRAFTS

Factor	Antigen	
	LCM virus	Mouse graft
1. Initial exposure must be close to or before birth	+	+
2. Lifelong tolerance to antigen can be acquired	+	+
3. Tolerance cannot be broken by other agents	+	+
4. Tolerance can be imitated by X-ray or cortisone treatment	+	+
5. Immune response increases with time interval between birth and exposure to antigen	+	+
6. Circulating antibody (neutralizing) found to be present in 5	−	−

This form of latent infection is quite different from other categories of latency in which the virus is partially suppressed by antibody, or exists in a resistant intracellular state. The critical point of difference lies in the lack of immunological reaction of the host in the neonatally induced latency; this type of latency constitutes an asymptomatic quasi-congenital infection

continuing apparently throughout the normal life span of the mouse. It is proposed to call this "vital" infection, this being a convenient mnemonic for "viral, immunologically tolerated acquired latent infection."

It is still necessary to account for the lethal disease resulting from postnatal infection with LCM virus. On the immunological basis postulated, the lesions caused by postnatal infection are due to the immune response of the host to the virus. This response is believed to cause damage to host cells, owing to one of the following mechanisms: (1) an allergic or hypersensitivity phenomenon; (2) a direct cytotoxic effect of antibody upon cells containing the viral antigen; (3) a cross reaction of antiviral antibody, to include host tissue. In the latter case, such cross-reacting antibody would probably be indistinguishable from "autoantibody". It is perhaps significant that a high degree of immunological tolerance can only be acquired by a host towards closely related antigens (5) which might be expected to cross-react with host tissue.

The criteria required to establish a "vital" infection can be summarized thus:

1. The virus is noncytopathogenic for the host.
2. The virus must be highly adapted to the host tissues.
3. The host must be infected with the virus before it is capable of immunological response.
4. The virus must be detectable by its ability to cause disease in a nontolerant host.

Discussion

The possibility that host response caused the symptoms of LCM in mice was entertained by Rowe (21, 22), who considered that the lymphocytic cellular reaction was probably responsible and that this was inhibited by X-irradiation. Homografts have been shown by Billingham *et al.* (6, 7, 5) and Cannon and Longmire (10) to be tolerated longer after suitable X-irradiation or cortisone treatment. During symptomatic infection with LCM it was shown that large amounts of soluble antigen occur in the infected tissue (25, 26), and also that antibody against it differed from virus neutralizing antibody (27, 28). Several workers found little or no neutralizing antibody during LCM infection although complement-fixing antisoluble substance antibodies appeared early in the disease (33, 28, 17, 21). This lack of neutralizing antibody in spite of virus disappearance is comparable to the inability of Billingham and Brent (4) to demonstrate serum antibodies as the operative agents in transplantation immunity.

That mice might in some cases become immunologically tolerant to LCM virus was first suggested by Burnet and Fenner (9) with respect to Traub's original findings; however, this concept does not otherwise seem to have been investigated further. Whitney (38) reported the lack of immediate clinical response of infant mice to intracerebral LCM virus inoculation, but did not investigate the latent aspects of the infection. Billingham *et al.* (5)

refer to Traub's work in this connection but consider that the tolerance shown by Traub's latently infected mice only applied to the "disease", that it was not specific immunological tolerance of the host to the virus. Whether other virus host systems can be classified as "vital" infections remains to be seen. Burnet (8) has suggested that human serum hepatitis may be an example of this type of tolerance. This concept appears to be supported by the lack of evidence of cytopathogenicity to human hepatitis viruses in vitro, which would also suggest a parallel between the pathogenesis of human viral hepatitis and murine LCM. In several respects equine infectious anaemia (*Trifur equorum*) is similar since it has been observed that infected horses may carry virus in their blood for years without being sick, although the blood can cause fatal illness in noncarriers (23).

The LCM titers of mouse brains reported here are lower than those found by other workers. This is apparently due to the strain of mice which was used since the same virus pool gave the same titer in Albany strain albino mice, but the brains of the latter gave comparable titers (in Albany mice) to those of other workers.

The unexpected response of intraperitoneally inoculated mice to subsequent X-irradiation appears to be an example of the accelerated response reported by Traub (33), Haas (17), and Rowe (21). This apparently occurs when a cerebral infection develops during a stage of partial immunity in the mouse.

It appears from the results reported here that the immunizing titer of the stock virus was higher than its infective titer. This observation has not been further investigated, but may be related to the finding of Smadel and Wall (27) that infected tissue contains relatively large amounts of soluble antigen, compared with the virus titer.

It is noteworthy that none of the methods used succeeded in causing overt symptoms of LCM to appear in mice with "vital" LCM. This contrasts with the induction of overt cerebral disease in latent "carriers" by Traub (31), who induced convulsive LCM in mice (which had been naturally infected with LCM), by the intracerebral injection of sterile broth. Haas (17) repeated this observation, using intracerebral cornstarch, in mice which had been given an inapparent infection by subcutaneous inoculation. This apparent conflict in results arises from the fact that there are two different conditions which were previously confused and grouped together under the term "latent infection". These are:

1. "Vital" infections which cannot be induced to cause overt disease.

2. Inapparent infections which can frequently be induced to cause overt disease. The mechanism of induction appears to involve damage to the blood-brain barrier in chronically infected partially immune animals.

The induction of convulsions following sterile intracerebral inoculations described by Traub and Haas is an example of inapparent infection (No. 2 above). "Vital" infection with LCM can be distinguished from latency due to chronic or inapparent infection by this difference in response to cerebral trauma.

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ABNORMAL GROWTH INDUCED BY PENICILLIN IN A STRAIN OF ALCALIGENES FECALIS[1]

K. G. LARK[2]

Abstract

The effect of penicillin on strain LB of *Alcaligenes fecalis* has been studied. In tryptone, 50 units/ml. of penicillin transforms the bacillary form of this organism into a protoplast-like structure (globular form) which is capable of exponential growth and division. The division rate of this globular form is about one-half that of the bacillary form. Removal of penicillin results in the reversion of the globular to the bacillary form. Multiplication of the globular form has been found to depend on the presence of some principle present in tryptone not required for bacillary growth in the absence of penicillin. The kinetics of the transformation and reversion process have been studied, leading to the hypothesis that penicillin affects some component within the cell, this component being concerned with cell division and the elaboration of the cell wall.

Introduction

Penicillin can be used to produce a controlled environmental change in a population of cells, bringing about a derangement of the division process and corresponding changes in the physiological and biochemical processes of the cell.

The production of abnormal *growth* forms of *Alcaligenes fecalis* in such an environment has been qualitatively described by Bonifas (2). A characteristic effect of penicillin in this system, as in others (4, 10), is the production of protoplast-like forms upon treatment of normal bacillary forms with penicillin.

Previous work on this (2, 5) and other systems (4, 10) has been concerned with loss of bacterial cell-wall material under the influence of penicillin.

This paper reports the development of a quantitative approach for studying the effects of penicillin on the cell-division process. This, used in combination with synchronization of cellular division, may lead to a clearer concept of the processes determining cellular division.

Materials and Methods

Bacterial Strain

A motile strain of *A. fecalis*, isolated originally from the Arve River, Switzerland, by Dr. V. Bonifas (2), was grown in the presence of penicillin and a stock prepared from a single colony taken from the surviving culture.* The growth properties of this derived strain, LB, have remained constant throughout these studies. Stocks of the strain were maintained in isolated colonies on tryptone-agar plates and used in preparing overnight tryptone cultures

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*Although this strain was originally believed (2) to belong to the genus *Proteus*, its fermentative characteristics have led us to classify it as *A. fecalis*.

for weekly use. Fresh agar plates were prepared monthly and stored in the refrigerator. Cultures for daily use were prepared from the overnight tryptone cultures.

Media

Two types of liquid media were used in all of the growth experiments described in this paper. The first, *tryptone* medium, consisted of 1% Difco tryptone + 2% NaCl at pH 7.2. The second, *defined* medium, was that described by Medill and O'Kane (6), containing 0.5% Difco vitamin-free casamino acids or 0.1% glutamic acid as a nitrogen source. One gram of NaCl was added to each 100 ml. of this medium. Viable counts were carried out on agar plates containing 1.5% Difco agar, 1% tryptone, and 0.5% NaCl. Top agar consisted of 0.7% Difco agar, 1% tryptone, and 2% NaCl. In all experiments the potassium salt of crystalline penicillin G (Lilly) was used.

Measurements of Culture Density

Viable colony counts were made by the agar layer technique (1). This technique was modified in that only the bacteria from a countable dilution were incorporated in the top layer agar. Turbidimetric measurements were done with a Meunier turbidimeter with a No. 59 filter. The maximum transmission of light, through this filter, occurs at 585 mμ. Microscopic counts of "crescent" forms were made in a Tuerk counting chamber, 0.1 mm. in depth, divided into mm.^{2}/400 and mm.^{2}/25 squares.

Cytological Observations

Cytological observations were made with a Wild M20 phase-contrast microscope (15× ocular, 87× oil immersion objective). Drops of liquid preparations to be photographed were placed on the surface of a gelatin film. (Obtained from photographic plates by fixation, washing in distilled water, and drying.) Immediately after the deposition of the culture drop, a cotton fiber was added and the drop containing the fiber was covered with a glass cover slip. In this manner, cells on the edge of the preparation were immediately trapped and crushed between the cover slip and the gelatin surface. On the other hand, cells next to the cotton fiber remained free in suspension, subject to Brownian motion. Cells midway between these extremes were quickly found, which were held uncrushed and undistorted, between the cover slip and the gelatin surface, permitting photography. The total time elapsing between removing a sample from the culture and photographing it was about 10 minutes.

Results

Initial experiments were concerned with the process of transformation of the bacillary form into the abnormal growth form in order to define conditions for reproducibility of future experiments.

Transformation to, and Growth in, the Abnormal or "Globular" Form

A. fecalis strain LB was grown with aeration at 37° in tryptone medium. In this medium the strain grows as a motile bacillary form with a generation

period of 40 minutes. Growth is exponential under these conditions, provided the density of the bacterial population does not exceed 4×10^8 cells/ml. In these experiments, bacterial densities were therefore restricted to the range of $3\times10^7 - 2\times10^8$ cells/ml.

When penicillin is added to an exponentially growing culture at a concentration of 50 units/ml., the individual cells undergo a striking cytological change into a budding globular form. This change takes place over a period of 150 minutes following the addition of penicillin progressing through the sequence of cytological changes shown in Fig. 6, A–G. Exponential growth of the globular forms may be maintained indefinitely by diluting the culture in fresh medium containing penicillin. When this is not done, the cells eventually reach a stationary phase of growth, characterized cytologically by extremely dense globular forms which are highly refractile when seen in the phase-contrast microscope (Fig. 6, I).

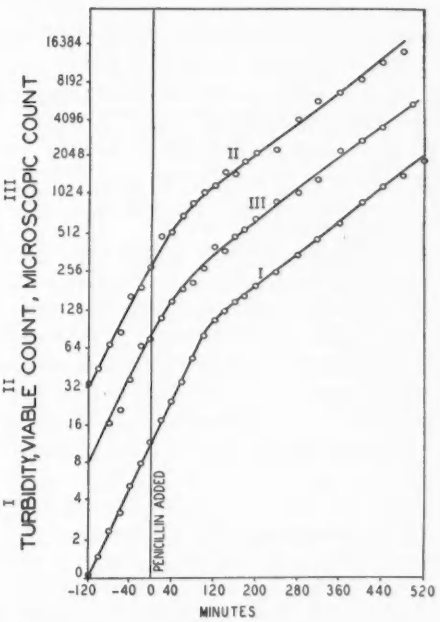


FIG. 1. Transformation in tryptone of the bacillary into the globular form of LB upon addition of 50 units/ml. of penicillin. Curve I, culture density measured turbidimetrically; Curve II, number of cells measured as viable colony-forming units; Curve III, number of crescents capable of arising from the population.

Crescents counted microscopically after a 3-hour incubation in synthetic medium + 50 units/ml. penicillin. The culture diluted 1/10 at the times indicated into the synthetic medium containing penicillin.

Ordinate in arbitrary units: At 0 minutes the values obtained experimentally were 116/cm., 2.8×10^8/ml., and 3.1×10^8/ml. for turbidity, viable colony counts, and crescent number, respectively.

After the addition of 50 units/ml. penicillin, the density of the culture continues to increase exponentially at an unchanged rate for a period of 2 hours. At the end of this period there is an abrupt drop in the rate of growth to a new value approximately one-half of the original rate (Fig. 1, Curve I). The culture then proceeds to grow exponentially at the new rate, provided the environmental conditions are maintained constant (continued dilution in tryptone containing penicillin).

On the other hand, the rate of cell division (measured by viable colony count) begins to decrease within 10 minutes after the addition of penicillin, gradually reaching a constant rate equal to about one-half the initial rate. Cell division is then maintained at a constant exponential rate, provided the environmental conditions are maintained constant.

The data in Curves I and II in Fig. 1 suggest that cell division is more immediately sensitive to the effects of penicillin than cell growth. This is not due to death of some of the cells in the population because normal bacillary cultures yield the same number of colonies when plated on penicillin and on normal tryptone agar, indicating that all of the cells remain viable after treatment with penicillin, despite characteristic changes in their cytology and division rate on penicillin agar. (Cells from colonies grown on penicillin plates appear cytologically as globular forms. Such colonies, produced in the presence of 300 units of penicillin, require 36–48 hours to develop as compared to the normal period of 18–24 hours.)

Reversion of the Globular to the Bacillary Form

When an exponentially growing culture of globular forms is centrifuged and resuspended in tryptone without penicillin, the cells return to their original bacillary form. This process covers three to four generation periods. In the process of reversion to the bacillary form, the cells pass through a bizarre morphological state, characterized chiefly by the highly branched character of the cells, with no differentiation into cell units observable in the phase-contrast microscope (Fig. 7, E). Normal bacillary forms first appear after about 4 hours. These forms do not dominate the population, however, until after about 7–8 hours.

A comparison of Curves I and II in Fig. 2 indicates that the effects of penicillin on cell division are more lasting than those on cell growth, the rate of the former remaining low for an appreciably longer period than the growth rate. Furthermore, the increase in the growth rate of cells is gradual, whereas the increase in cell-division rate is more abrupt. These data are consistent with the cytological character of the reverting cells (Fig. 7, E) which increase in size without simultaneously undergoing division into smaller bacillary units.

pH Sensitivity of Growth and Multiplication in the Globular Form

The formation and growth of globular forms is extremely sensitive to the acidity of the medium.

In the experiments reported above the pH was maintained at 7.2. When penicillin is added to a culture growing in the bacillary form in a tryptone

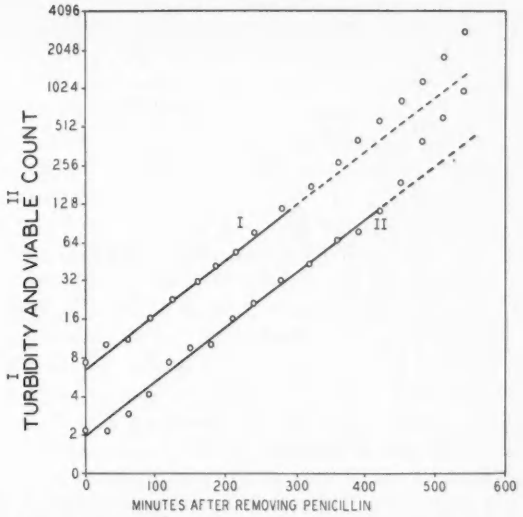


FIG. 2. The reversion of the globular to the bacillary form of LB following the removal of penicillin. Globular forms were growing exponentially in tryptone containing 50 units/ml. penicillin prior to the removal of penicillin at 0 minutes by centrifugation. Curve I, culture density measured turbidimetrically; Curve II, number of cells measured as viable colony-forming units.

Ordinate in arbitrary units: At 0 minutes the values measured experimentally were 7.5/cm. and 1.05×10^7/ml. for turbidity and viable colony counts, respectively.

medium at *pH 6.8*, the cells develop as previously shown (Fig. 6, A–F) but instead of separating into smaller globular forms, the cells continue to enlarge into forms of the type shown in Fig. 7, G. In such cultures turbidity increases for a period of 1–2 hours and thereafter remains constant. In contrast, cell division appears to cease within a few minutes after the addition of penicillin, although a constant colony count indicates that the cells remain viable and that a bacteriostatic situation is in effect. An alkaline environment (pH 7.4–8.0) has a less drastic effect, in that globular forms develop, grow, and divide, but at a reduced rate equal to two-thirds to one-half the rate at pH 7.2.

Nutritional Requirements for Growth in Globular Form

Strain LB will grow in a defined medium (containing lactate as an energy source and casamino acids as a nitrogen source) as a motile bacillary form with a generation time of 40–45 minutes at 37°. If penicillin is added to such a culture in concentrations of 50 units/ml., the culture undergoes the cytological transformation shown in Fig. 7, A–D. This transformation results in the conversion of all of the cells into large (3–5 μ in diameter), spherical forms in which the protoplasm of the cell appears to be confined to a limited portion of the surface of the spheres. Viewed in two dimensions, this results in a crescent-shaped distribution of the protosplam within the cells. We shall refer to such forms as *crescents*.

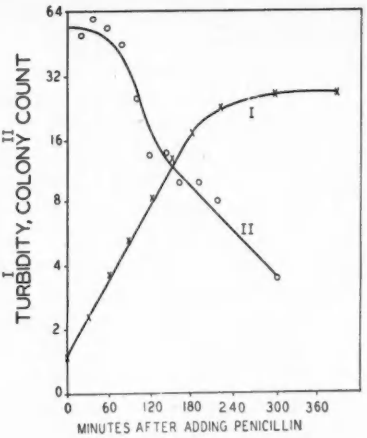


FIG. 3. Transformation of the bacillary into the crescent form of LB in synthetic medium containing 50 units/ml. of penicillin. Curve I, culture density measured turbidimetrically; Curve II, number of cells measured as viable colony-forming units. Ordinate in arbitrary units: At 0 minutes the values obtained experimentally were 15.5/cm. and 5.3×10^7/ml. for turbidity and colony counts, respectively.

Crescents are incapable of continued growth and multiplication. From Curve I of Fig. 3 it is seen that following a period of 4–5 hours, after the addition of penicillin, all growth ceases and no further increase in turbidity is found. As seen in Curve II, after a period of approximately an hour the number of viable colony-forming cells in the culture begins to decrease, the rate of inactivation becoming slower after approximately 60–70% of the population has been inactivated. Microscopic controls have revealed that this inactivation is not due to lysis, although lysis may occur later if such a culture is left overnight with continued aeration and incubation at 37°.

The striking difference observed between growth in the globular form and transformation to crescents appears to be related to some unknown substance present in the tryptone medium.* That this is the case may be seen in an experiment in which a culture of LB was grown in liquid media, to which tryptone had been added in concentrations of 2% to 0.1% to synthetic media containing 50 units/ml. penicillin. Quantitative growth in the globular form was obtained with tryptone concentrations of 0.5% or higher, whereas quantitative transformation of the population into crescent forms was obtained at lower concentrations. This eliminates the possibility that transformation to the crescent form and subsequent death of the culture is due to a toxic factor present in the synthetic medium.

*The substance present in tryptone appears to be insoluble in organic solvents and resistant to 1 *N* HCl at 100° for 1 hour. It is destroyed by heating in 6 *N* HCl at 108° for 5 hours. From this it would appear that the substances involved are not all simple substances, such as amino acids or purines and pyrimidines. Preliminary attempts to purify the principle by means of column chromatography have indicated that this approach is feasible and that more than one substance may be involved.

One of the most striking features of the effect of tryptone is the lack of a dose–response curve. The results obtained above for liquid cultures have been found to hold true independent of the bacterial concentration used for the inoculum, i.e., 0.25% tryptone will not prevent the transformation of either 10^5 or 10^8 cells into crescent forms, whereas 0.5% allows growth in the globular from inocula of 10^5 or 10^8 cells up to concentrations of $5 \times 10^8 - 10^9$ colony-forming units.

Transformation into the crescent form may be initiated, from either the bacillary or globular form. Samples were taken from a tryptone culture of LB both before and after addition of 50 units of penicillin—i.e., when cells were in the bacillary or globular form, respectively — and diluted 1/10 into synthetic medium containing 50 units/ml. of penicillin. (Globular forms will revert to the bacillary form in synthetic medium containing no penicillin.) These samples were then allowed to incubate for 3 hours, after which time the number of crescents present in each sample was counted. Curve III in Fig. 1 presents these counts as a function of the time at which the sample was taken from the tryptone culture, i.e., it measures the ability of both bacillary and globular forms to form crescents. The ability to form crescents appears to be the same for both bacillary and globular forms when measured per cell-division (colony-forming) unit, Curves II and III being essentially parallel. On the average, a little more than one crescent is formed from each colony-forming unit (see legend to Fig. 1).

Effects of Penicillin Concentration upon the Transformations of Strain LB

Penicillin concentrations below *30* units/ml. have no effect on tryptone cultures of LB which continue to grow exponentially in the bacillary form at a slightly reduced rate. On the other hand, concentrations of penicillin above *100* units/ml. result in the death of the culture.*

The lethal effects of penicillin on a tryptone culture of LB have been followed in detail. Cytologically, following the addition of 300 units/ml. penicillin, the cells are rapidly transformed (during a 50 minute period) through the stages shown in Fig. 6, A–G, developing eventually into large grape-like clusters of protoplasm which lyse, leaving empty membranes, Fig. 7, F. Following a small increment in turbidity, lysis leads to a progressive clearing of the culture. Colony counts have demonstrated the immediate onset of inactivation of the cell population following the addition of penicillin.

The rate of formation of crescents in synthetic medium is similarly dependent upon penicillin concentration, although the cytological sequence remains the same at all concentrations above 35 units/ml. Penicillin concentrations below 35 units/ml. are insufficient to transform the population into crescent forms, a few such forms (about 5% of the population) being found at 27.5 units/ml., while concentrations higher than 35 units/ml. transform the entire population. The rate of transformation increases with increasing penicillin concentration.

*These results are only true for liquid media. On tryptone agar, concentrations of 300 units/ml. penicillin are necessary to produce colonies of globular forms whereas concentrations of 1000 units/ml. are required to destroy colony formation. Three hundred units per milliliter suffices to inhibit all colony formation on synthetic agar.

The transformation of bacillary into crescent forms has been followed quantitatively as a function of the penicillin concentration in two types of experiments. In the first, cultures of the bacillary forms of LB growing exponentially in defined medium were subjected to different concentrations of penicillin for varying lengths of time. After exposure to penicillin, the cultures were diluted in synthetic medium to a penicillin concentration of 5.3 units/ml. and incubated for a period of 3 hours. At this time formol, which fixes both bacillary and crescent forms, was added to a concentration of 1% and the number of crescents present were counted.

This experiment serves to determine the rate at which penicillin alters the bacillary cell in such a manner as to result in its *eventual* transformation into a crescent form, despite the reduction of the penicillin concentration to levels well below 25 units/ml. The results of this experiment are presented in Fig. 4.

In a second type of experiment the rate of the *actual* transformation of the bacillary forms into crescent forms was measured. This was done as follows: osmium tetroxide, which normally fixes the bacillary form of LB, lyses the crescent or globular forms of this strain. Cells being transformed into glo-

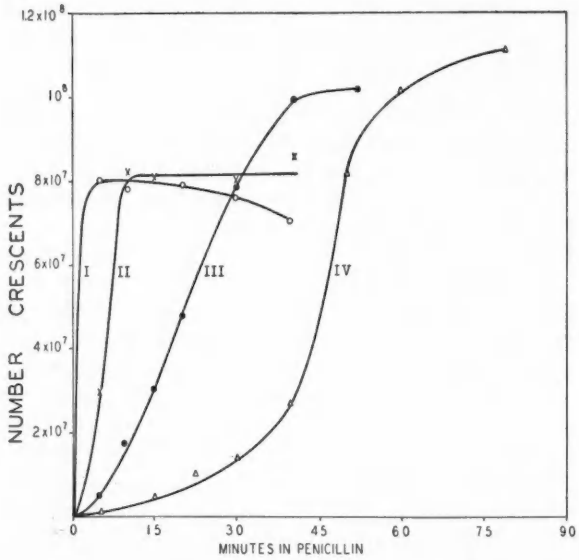


FIG. 4. Induction of crescent formation as a function of the length of time in penicillin and of the penicillin concentration. Curve I, 320 units/ml. penicillin; Curve II, 160 units/ml. penicillin; Curve III, 80 units/ml. penicillin; Curve IV, 40 units/ml. penicillin. Penicillin concentrations below 30 units/ml. fail to induce crescent formation in a growing bacillary population.

Ordinate: The number of crescents formed during a 3-hour incubation period after the removal of penicillin. At 0 minutes there were 8.3×10^7 ml. colony-forming units present in the population.

bular or crescent forms are susceptible to osmium lysis as soon as they reach the form indicated by the arrow in Fig. 6, C. Utilizing this fact, cultures of 4B growing exponentially in synthetic media were subjected to various concentrations of penicillin ranging from 40 to 320 units/ml.; 4-ml. samples were removed at intervals and added to 0.2 ml. of 2% OsO_4. Under these conditions, all cells which were still in the bacillary form were fixed and they remained in this form. All forms which had progressed up to or past the stage shown by the arrow in Fig. 6, C, were lysed. At the end of the experiment all samples were read turbidimetrically. The results of this experiment are shown in Fig. 5.

The ability of penicillin to affect cells is a function of the penicillin concentration (Figs. 4 and 5). With increasing doses of penicillin, the rate at which cells become induced to undergo an eventual transformation into crescent forms increases, as does the rate of actual transformation. Furthermore, the rate of crescent induction may be seen to increase out of all proportion to the increase in penicillin concentration.

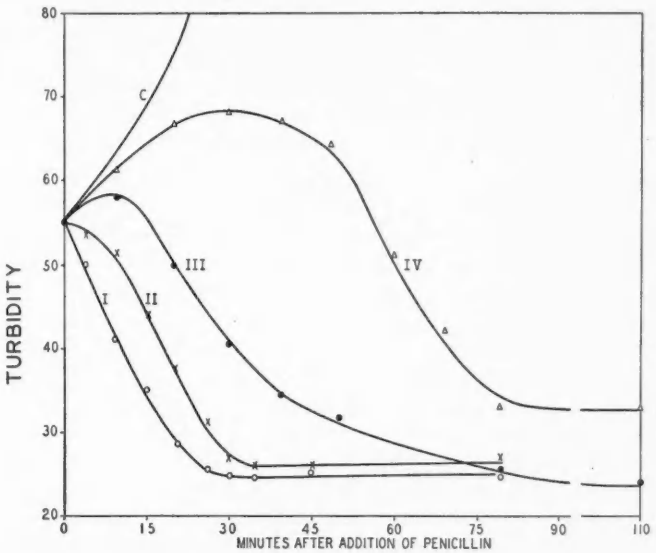


FIG. 5. The formation of crescents as a function of penicillin concentration and the length of time in penicillin. Curve I, 320 units/ml. penicillin; Curve II, 160 units/ml. penicillin; Curve III, 80 units/ml. penicillin; Curve IV, 40 units/ml. penicillin; Curve C, the turbidity of a culture developing in the presence of 50 units/ml. penicillin not subjected to osmium lysis.

Crescent formation is measured by the addition of 0.2 ml. of 2% OsO_4 to 4 ml. aliquots taken at intervals from the cell population. Under these conditions the degree of crescent formation is represented by the degree of lysis of the culture.

The viable colony count at the start of this experiment was 1.6×10^8 cells/ml.

A comparison of the results presented in Figs. 4 and 5 indicates that at all penicillin concentrations, the rate of transformation of cells into the crescent form is slower than the ability of the penicillin to affect the cells, i.e., the lysis in Fig. 5 lags behind the increase of crescents in Fig. 4 by about 20 minutes.

The Effect of Tryptone or Synthetic Medium during Induction on the Process of Eventual Transformation into Globular or Crescent Forms

In the experiments just discussed, it was seen that transformation into the crescent form may take place in the absence of inducing concentrations of penicillin. This finding has been utilized to determine whether the presence or absence of tryptone *during the period of induction* determines whether the outcome of the transformation will be globular or crescent in form.

Exponentially growing cultures of LB, in both synthetic and tryptone media, were subjected to 300 units/ml. of penicillin for 5 minutes, and following induction each culture was diluted 1/60 into either tryptone or synthetic media. The resulting four cultures were then examined microscopically for the production of globular or crescent forms. Only those cultures which were allowed to develop in tryptone following induction were transformed into globular forms (which later reverted to bacillary forms); whereas those developing in synthetic medium were transformed into crescents. This occurred whether induction had taken place in the presence of tryptone or synthetic media. Thus, penicillin exerts the same inducing effect on LB in the presence or absence of tryptone, the latter serving, by its presence during the transformation process, to determine what type of transformation the cell will undergo.

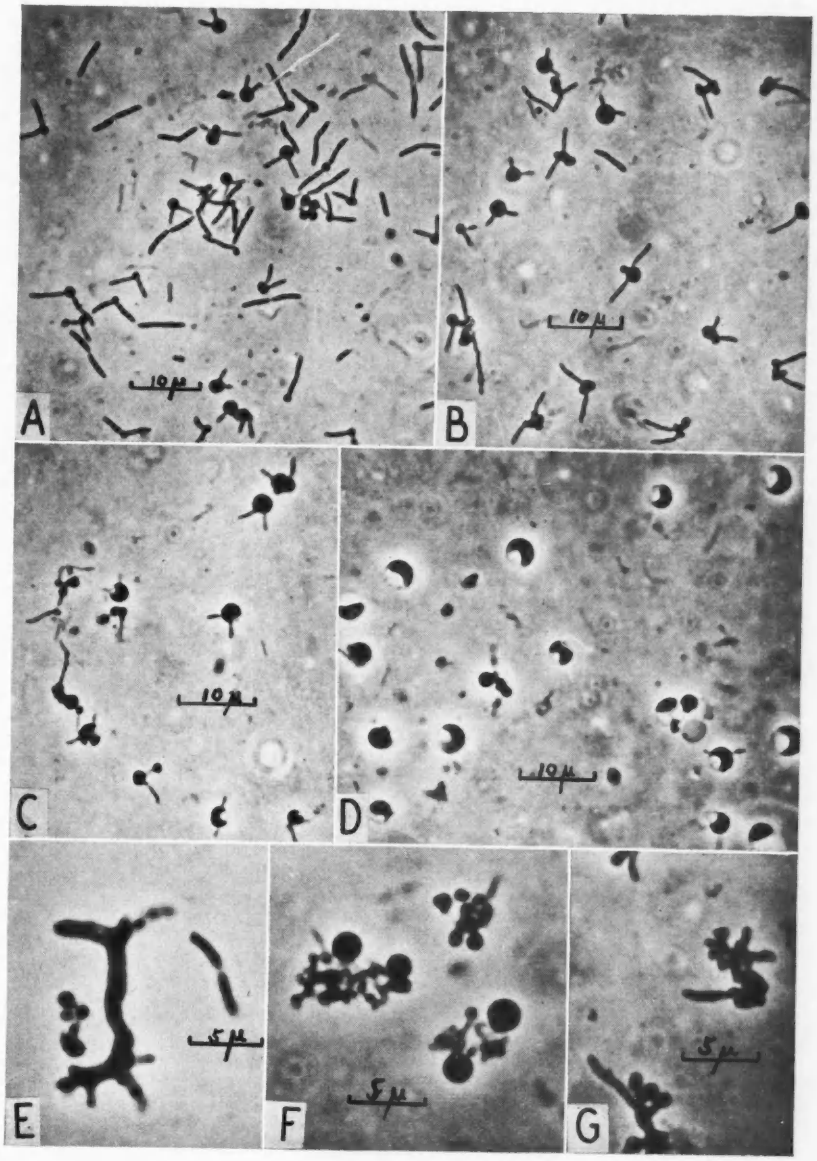
Discussion

The point of departure in this investigation was the observation that strain LB of *A. fecalis* undergoes a cytological transformation in the presence of penicillin from a bacillary into a globular form and continues to grow in this latter form as long as penicillin is present (2).

The recent investigations of Strominger (8) on the biochemistry of, and Murray *et al.* (7) on the cytology of the cell walls of staphylococci treated with penicillin have shown that penicillin blocks the formation of new cell-wall material. Similar results have been recently obtained by Weidel and Primosigh (10), who have shown that the cell walls of Gram-negative organisms possess a component, similar in composition to the cell wall of Gram-positive organisms, the synthesis of which is blocked by penicillin. Previous discussions of penicillin-induced globular forms in Gram-negative organisms have pointed out (2, 5) that in view of their cytological appearance and physical fragility, these forms would appear to possess cell walls with altered physical

FIG. 6. Transformation of the bacillary into the globular form of LB in the presence of tryptone and 50 units/ml. penicillin. × 1350
(A)–(G) Time after adding penicillin: (A) 0 minutes; (B) 20 minutes; (C) 40 minutes; (D) 60 minutes; (E) 80 minutes; (F) 100 minutes; (G) 140 minutes.
(H) Globular form of 4B. × 2700
(I) Appearance of cells in an overnight culture of globular forms. × 2700

PLATE I



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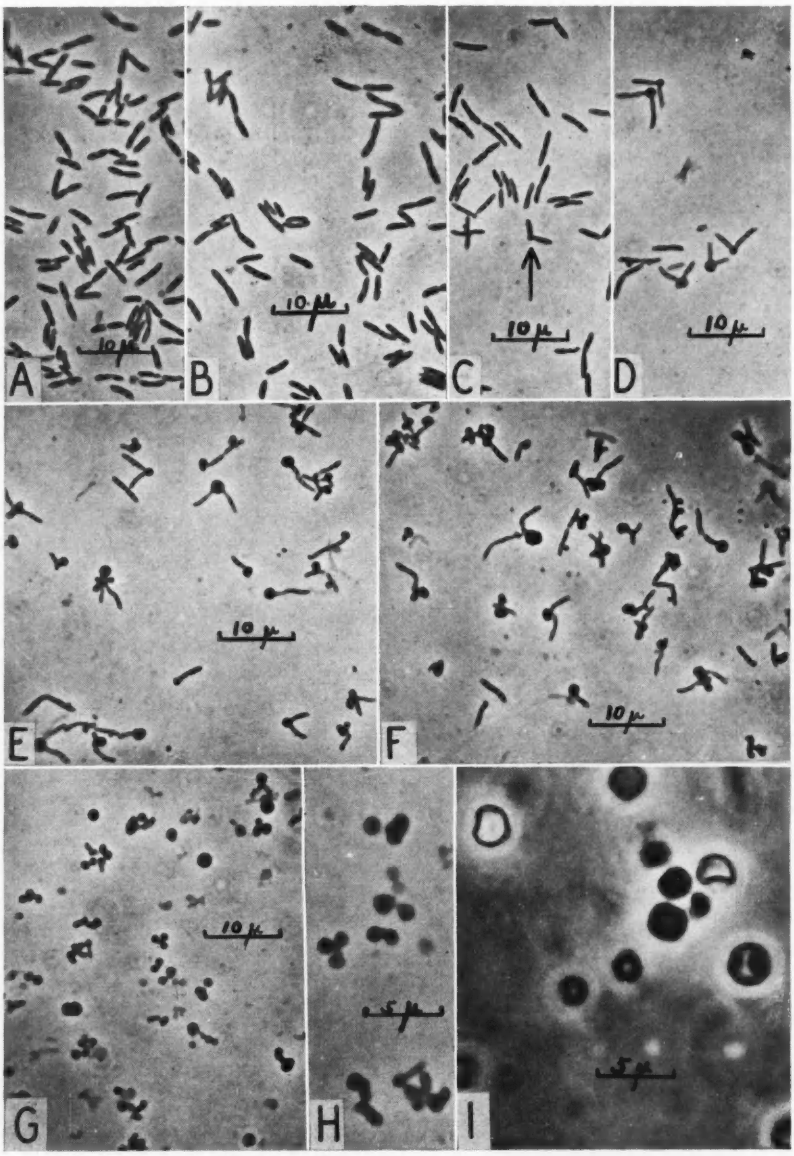
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characteristics.* The susceptibility of the globular and crescent forms of strain LB of *A. fecalis* to lysis by changes in osmotic pressure, or by the addition of OsO_4, together with the pH sensitivity of growth in the globular form, indicate that these forms also possess an altered cell wall, presumably lacking some essential constituent.

The system which has been studied in these experiments presents an outstanding and perhaps unique characteristic. This lies in the ability of *A. fecalis* to be transformed quantitatively into globular forms, under the influence of penicillin, and the ability of these forms to *grow* exponentially in the presence of penicillin, retaining their characteristic morphology. This property has enabled us to carry out detailed studies of the growth properties of these forms.

As was pointed out, the globular forms of strain LB appear to lack a portion of their cell wall. Nevertheless, these forms are able to retain a sufficient quantity of the various coenzymes and other low molecular weight metabolites necessary for the processes of growth and division. (It is inconceivable that tryptone should contain all of the metabolites essential to the life and division of the cell.) From Figs. 1 and 2, Curves I and II, a difference may be seen between the progress of cell growth as measured turbidimetrically and of cell division as measured by viable colony counts. During the transformation of bacillary forms into globular forms, it was found that cell division was affected before any effect was noted on cell growth. Conversely, during the reversion of cells from the globular to the bacillary form, an increase in cell-growth rate was noted prior to that in cell-division rate. This result is in contrast to that normally observed in cells depleted of essential metabolites, such as in "old" cultures, or in cultures starved of essential metabolites where cell division proceeds for a considerable period after cell growth has ceased.

These results have indicated a primary effect of penicillin on the cell-division process. This idea is greatly strengthened by the observed lack of cell division of LB when growing in the presence of penicillin in synthetic medium. Under these conditions, it would appear that this organism requires

*Lederberg (4) has drawn attention to the similarities between forms of the type described here and the protoplasts produced when Gram-positive organisms are treated with lysozyme (9). Since the mechanism by which penicillin produces these forms differs from that whereby lysozyme produces the protoplasts of *B. megaterium* and is, in fact, more similar to the direct action of penicillin on Gram-positive cells, we would prefer to continue to refer to the abnormal growth forms of *A. fecalis* as globular or crescent forms, retaining for the present a purely descriptive terminology (cf. (10)).

FIG. 7. Transformation of the bacillary into the crescent form of LB in synthetic medium containing 50 units/ml. penicillin. × 1350

(A)–(D) Time after adding penicillin: (A) 50 minutes; (B) 80 minutes; (C) 120 minutes; (D) 180 minutes.

(E) Stage in the reversion of the globular to the bacillary form of LB 230 minutes after removal of penicillin. × 2700

(F) Effect of 300 units/ml. penicillin on a tryptone culture of LB; 120 minutes after adding penicillin. × 2700

(G) The effect of acidic pH on the penicillin induced cell. Culture in tryptone medium, *pH 6.8*, 180 minutes after the addition of 50 units/ml. penicillin. × 2700

some additional nutritional factor for cell division not required for division and growth in the normal bacillary form in the absence of penicillin.

The role of this growth factor would appear to be more complex than that of an essential metabolite as no evidence of a dose–response curve has been obtained. Experiments in which induction in tryptone medium resulted in the transformation into crescents of cells afterwards placed in synthetic medium indicate that the cell is unable to concentrate and retain the substance present in tryptone. A possible explanation of these results may be that this substance exerts a catalytic effect of some type in mediating the type of damage which penicillin produces in the cell. The threshold concentration would represent that concentration of tryptone which assures a sufficient concentration of the necessary substance within the cell, for such catalytic action to be effective.

The demonstration that tryptone acts on the transformation process rather than on the actual induction by penicillin serves to clearly delineate the process of penicillin *induction* from that of *transformation*. Furthermore, the data in Figs. 4 and 5 would indicate that a period of some 20 minutes elapse between induction of the cell and the appearance of the first visible manifestations of the transformation. Future experiments with ultrathin sections may demonstrate whether any cytologically discernible changes within the cell take place during this period.

It is interesting to note the high degree to which the rate of crescent induction is dependent on the penicillin concentration in the medium. Thus (Fig. 4) at 5 minutes, 1.5, 7, 36, and 100% of the cells are induced to form crescents in 40, 80, 160, and 320 units of penicillin respectively. From this it follows that several molecules of penicillin must interact with the cell in order for the cell to be induced to undergo transformation into the crescent form. This conclusion is strengthened by the finding that there is a threshold in penicillin concentration (between 27.5 and 35 units/ml.) below which penicillin is not able to transform the cell. Previous work with radiopenicillin (3) has indicated that several molecules of penicillin may be irreversibly bound by the cell. From the data above, it would also appear that several penicillin molecules are necessary to produce a *biological* effect on the cell.

In all of the experiments which have been carried out, no indication of induction to partial transformation has been found. Either cells are induced to undergo complete transformation into crescents, or else they remain in the bacillary form. This observation together with the observed threshold concentration for penicillin action suggests that the action of penicillin is not of a non-specific toxic nature, but is, perhaps, directed against some specific cell component whose functioning, necessary to cell growth and division, is destroyed.

Acknowledgments

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Dr. Kellenberger, whose guest I was while in Geneva. The stimulated discussions, helpful assistance, and encouragement which Dr. Kellenberger gave to me at all times were in large part responsible for making this work possible. I wish to thank Mme Kellenberger, Mlles Tine Bonne and Janine Sechaud, Dr. V. Bonifas, and M. Werner Arber for the helpful discussions which were an indispensable part of my stay in the Laboratory of Biophysics.

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VARIATION DURING THE CELL-DIVISION CYCLE IN THE PENICILLIN INDUCTION OF PROTOPLAST-LIKE FORMS OF ALCALIGENES FECALIS[1]

K. G. LARK

Abstract

Experiments have been carried out on the penicillin-controlled induction of protoplast-like forms, crescents, in synchronized cultures of a strain of *Alcaligenes fecalis*. The results have shown that during the cell-division cycle the cell passes through a stage shortly before division during which it is less easily induced to form crescents than at other periods in the division cycle. The data presented indicate that this is not due to a transitory period in the division cycle during which the cell is refractory to penicillin. The findings are discussed in support of the hypothesis that penicillin exerts its effect by destroying some component within the cell whose functioning is necessary for further cell-wall synthesis.

Introduction

Experiments utilizing cell-free extracts of bacteria, and radioactive penicillin, have demonstrated the presence of a component in bacteria capable of combining irreversibly with penicillin (2). The recent biochemical work of Park and Strominger (9) and the cytological observations of Murray *et al.* (8) have demonstrated that a major effect of penicillin is to block the formation of new cell-wall material. These findings have served to explain the formation of protoplast-like forms of certain Gram-negative bacilli resulting from the action of penicillin (3, 1, 6, 4).

A gap in our knowledge exists, however, in considering what occurs in the cell between the irreversible binding of penicillin and the eventual cessation of cell-wall synthesis. Results of experiments on the irreversible penicillin induction of protoplast-like forms have suggested that in inducing the transformation of a cell into a form lacking a cell wall (crescent), penicillin acts by destroying some specific component within the cell, whose functioning is vital to the normal growth of the cell (4).

In cells containing a single penicillin-sensitive component, destruction of the component would result in the induction of crescent formation. On the other hand, cells containing two such components should not be induced to form crescents until ***both*** components ceased to function. If the inactivation of these components occurs independently within a cell it might be expected that the probability of a given dose of penicillin inducing crescent formation would be less in cells containing two than in cells containing a single such component. It should, therefore, be possible to measure the frequency of the latter type of cells by measuring the decrease in frequency of crescent formation ensuing from a given inducing dose of penicillin. In order to study this experimentally, it is necessary to deal with a population

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Contribution from the Department of Microbiology, Saint Louis University School of Medicine, St. Louis, Missouri, U.S.A. This work was supported by Grant No. E-1391 of the National Institutes of Health, Bethesda, Maryland, U.S.A.

of cells in which an increase in the number of penicillin susceptible components may reasonably be expected to occur. Such is the case in populations of cells whose division cycles have been synchronized, since during the division cycle every component of the cell must be duplicated.

The present paper is concerned with the induction of protoplast-like forms (crescents) by penicillin in synchronized cultures of *Alcaligenes fecalis* strain LB, a system ideal for such studies since the induction of crescents is under the complete control of the experimenter and may be easily and quantitatively measured.

Materials and Methods

The characteristics of strain LB and its transformation into protoplast-like forms (crescents) were described in a previous paper (4). The media, reagents, and methods of enumeration (cells by colony counts and crescents by microscopic examination) have also been described.

Cultures were synchronized by a modification of the filtration technique described by Maruyama (7). This technique is based on the physical selection of large predivision cells from the rest of the population. The cultures, 200 to 100 ml., usually containing 2×10^8 cells/ml., were centrifuged and resuspended in 1 to 4 ml. of synthetic medium (4) from which casamino acids had been omitted. The resuspended cells were then placed under suction on a filter paper column consisting of, from top to bottom, one layer of Whatman No. 1 paper and four layers of Eaton Dikeman 624-23 paper. Following adsorption of the cells to the filter paper column, the column was washed under suction with 25 to 125 ml. of the basal mixture (depending on the volume and density of the original culture used). The entire process of adsorption and washing covered a period of 2 to 3 minutes. Immediately following the washing, the topmost layer of Eaton Dikeman paper was eluted in 10 to 80 ml. of the basal mixture at 37° and incubated for 20 minutes with aeration.* At the end of this time, a solution of 10% casamino acids was added to the culture to a concentration of 0.5% to initiate synchronized growth of the culture. As a result of this procedure, 10 to 80 ml. cultures containing $1–2\times10^8$ bacteria/ml. were obtained in which the division cycles of the individual cells had been synchronized. These cultures remained in synchrony for two cycles, a division occurring in the period from 35–45 minutes and 75–85 minutes following the addition of casamino acids. During these periods, about 75 to 80% of the cells divided, the rest dividing over the 30-minute interim periods. The generation time of such synchronized cultures was the same as that found in exponentially growing cultures, 40 minutes.

Induction experiments of two types were carried out—short-term and continuous. A description of the basic induction type experiment has been

*Due to the tendency of this strain to form chains of two to four cells, the synchrony of these cultures is blurred to a certain extent. This problem was alleviated by increasing the suction pressure used in the initial filtration and *discarding the topmost Whatman No. 1 paper* Although this decreased the yield of synchronized cells, it succeeded in removing cell chains from the culture at the onset of the experiments.

given previously (4). In the short-term experiments, aliquots of cultures were subjected to a high concentration of penicillin for a short period of time, and then diluted to a penicillin concentration which was ineffective in inducing crescents (5 to 10 units/ml.). In the continuous experiments, penicillin at a concentration of 40 units/ml. was added to the culture and samples diluted at intervals to ineffective penicillin levels. In both cases, the samples obtained after dilution were allowed to incubate for 3 hours after which they were fixed in formol, and the number of crescents formed counted.

Results

Short-term Induction Experiments

A series of experiments were carried out to ascertain the number of crescents induced by a given dose of penicillin at different periods of the cell-division cycle.

Aliquots of cells were taken at intervals from a synchronized culture and subjected to short-term inducing doses of penicillin consisting of either 200 units/ml. for 4.5 minutes or 400 units/ml. for 2.5 minutes. These doses were such as to induce transformation of 40–60% of the cells in an exponentially increasing population. The results of a typical experiment are shown in Fig. 1. As may be seen, shortly before cell division takes place, there is a reduction in the number of transformations effected to about 70% of the number found during the preceding portion of the cell-division cycle. During cellular division the counts of crescents return to their expected value.

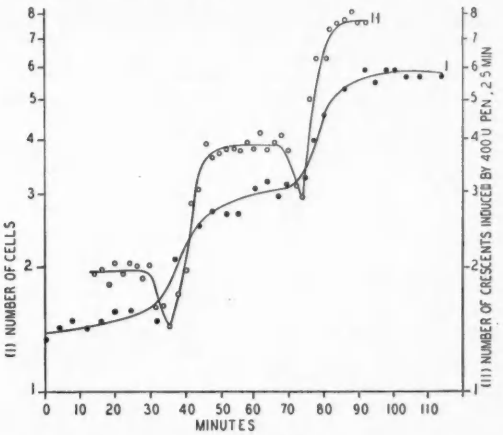

FIG. 1. Crescent inducibility of a synchronized culture at various periods of the cell-division cycle.

Curve I: Cellular division in the synchronized culture measured as colony counts. Ordinate, colony counts; one unit equals 10^8 colony-forming units per ml. Abscissa, minutes after initiation of synchrony by adding 0.5% casamino acids.

Curve II: Number of crescents induced in aliquots of the synchronized culture by addition of 400 units/ml. of penicillin for 2.5 minutes. Ordinate, crescents induced; one unit equals 4.0×10^7 crescents/ml. Abscissa, time in minutes, after initiation of synchrony in parent culture, at which aliquots were subjected to short-term inducing doses of penicillin.

Although these results are exactly what one expects to find if penicillin acts by destroying some cellular component which is normally duplicated immediately prior to cell division, the results obtained may also be accounted for by assuming a transient insusceptibility of the cell to penicillin (e.g., due to a short period of impermeability to penicillin) occurring immediately prior to cell division. It appeared possible to differentiate between alternatives if some means could be found to block cell division, while still allowing duplication of most cell components, including the postulated component sensitive to penicillin. Previous experiments indicated that such a system might be available in the use of low penicillin concentrations (40 units/ml.) barely sufficient to affect the transformation of normal to crescentic forms.

Ability of Penicillin to Block Cell Division

That the continued presence of penicillin at a concentration of 40 units/ml. suffices to block cell division has been shown in two types of experiments. Penicillin at a concentration of 40 units/ml. was added to aliquots taken from a synchronized culture at 0, 20, 40, and 60 minutes after the initiation of synchronized growth by the addition of 0.5% casamino acids (see Materials

TABLE I

THE EFFECT OF 40 UNITS/ML. PENICILLIN UPON CELL DIVISION IN SYNCHRONIZED CULTURES OF LB

Time after onset of synchronized growth that penicillin was added							
0 minutes		20 minutes		40 minutes		60 minutes	
Sample time, min.	Colony count $\times 10^5$	Sample time, min.	Colony count $\times 10^5$	Sample time, min.	Colony count $\times 2 \times 10^5$	Sample time, min.	Colony count $\times 2 \times 10^5$
1	511	20	472	39	448	59	540
6	512	25	498	47	520	67	505
11	467	30	574	54	510	75	606
16	479	35	546	62	473	83	758
21	447	40	562	70	525	91	890
26	465	45	652	78	533	99	909
31	422	50	831	86	508	115	804
36	419	55	763	94	468	125	814
41	460	60	770	102	521		
46	467	65	869	120	489		
51	440	70	799				
56	465	75	729				
61	453	80	726				
66	518	85	740				
71	520	90	710				
76	410	95	640				
81	448	100	614				

NOTE: Penicillin was added to aliquots of a synchronized culture of *A. fecalis* LB at 0, 20, 40, and 60 minutes. Colony counts were obtained at the various sample times by diluting 0.1 ml. samples into 10 ml., 0.1 ml. of this dilution into 20 ml. and plating 0.2 ml. of the last dilution for the 0 and 20 minute aliquots; 0.1-ml. samples of the last dilution were plated for the 40 and 60 minute aliquots.

and Methods) and the ensuing course of cell division in each aliquot followed by colony counts. As may be seen (Table I) the samples into which penicillin was introduced at *0* and at *40* minutes showed no increase in colony count. On the other hand, 60–70% of the cells in the samples into which penicillin had been introduced at *20* and at *60* minutes divided at 35 and 75 minutes, respectively (the times at which division normally occurs). In these cultures no second cycle of division, expected in normal cultures at 75 and 115 minutes respectively, took place.

In a second experiment, 40 units/ml. penicillin was added to aliquots taken from a synchronized culture at short intervals during the periods from 0–33 and 40–73 minutes after the initiation of synchronization and the two sets of aliquots titered for colony-forming particles—the former at 60 minutes, and the latter at 100 minutes.

In Fig. 2, Curves I and II, the results have been represented by plotting the number of colonies found at 60 or 100 minutes (15 minutes after the *end* of each expected division period) as a function of the time at which penicillin was added. (If no penicillin had been added to the various aliquots Curves I and II would consist of two horizontal lines whose ordinate values would

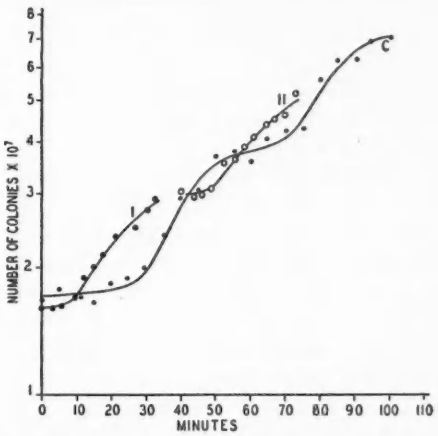

FIG. 2. The effect of the continued presence of 40 units/ml. penicillin on the course of cell division in a synchronized culture.

Curve C: Cellular division in the synchronized culture measured as colony counts. Ordinate, colony-forming units. Abscissa, minutes after initiation of synchrony by addition of 0.5% casamino acids.

Curve I: Colony-forming units found at 60 minutes following addition of 40 units/ml. penicillin to aliquots of the synchronized culture at various intervals prior to 60 minutes. Ordinate, colony-forming units found at 60 minutes (counts are 11% lower than Curve C owing to dilution by addition of penicillin). Abscissa, time in minutes, after initiation of synchrony, at which aliquots were mixed with 40 units/ml. penicillin. Penicillin remained in each aliquot until the time of sampling, 60 minutes.

Curve II: Colony-forming units found at 100 minutes following addition of 40 units/ml. penicillin to aliquots of the synchronized culture at various intervals prior to 100 minutes. Ordinate, colony-forming units found at 100 minutes (counts are 11% lower owing to dilution by addition of penicillin). Abscissa, as in Curve I. Penicillin remained in each aliquot until the time of sampling, 100 minutes.

be 3.3×10^7 and 6.6×10^7, respectively.) When penicillin is added between 30 and 40 minutes prior to division no cellular division takes place. On the other hand addition of penicillin for shorter periods of time preceding the expected division results in a progressively greater probability of cell division.

Continuous Induction Experiments

When penicillin, at a concentration of 40 units/ml., is added to an exponentially growing, non-synchronized culture of LB, there is a period of 35–40 minutes during which crescent induction proceeds very slowly and which is followed by a rapid increase in the number of such forms induced (4). Under these conditions (continuous induction), the effect of penicillin in inducing the individual cell appears to be cumulative, induction being completed in most cells between 40 and 50 minutes after the addition of penicillin.

If this cumulative effect of penicillin is upon some penicillin sensitive component of the cell, the production during the cell-division cycle of a "healthy" component in the cell should result in a prolongation of the time necessary for penicillin to induce the cell, the cell being induced when the new component is either destroyed by penicillin or removed from the cell unit by cell division. Synchronized cell populations in which "healthy"

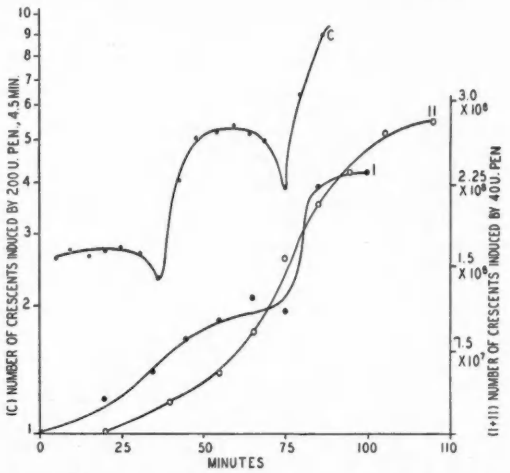

FIG. 3. Continuous induction of crescents by 40 units/ml. penicillin. Penicillin added to aliquots of a synchronized culture at 0 and 20 minutes after the onset of synchronized growth.

Curve I: Rate of crescent induction by 40 units/ml. penicillin, added at 0 minutes, as a function of the length of time penicillin has been in contact with the cells. Ordinate, number of crescents induced (linear scale). Abscissa, time at which penicillin was removed by dilution. (Penicillin added at 0 minutes.)

Curve II: Rate of crescent induction by 40 units/ml. penicillin, added at 20 minutes, as a function of the length of time penicillin has been in contact with the cells. Ordinate, as in curve I. Abscissa, as in curve I. (Penicillin added at 20 minutes.)

Curve C: Crescent inducibility of the parent culture from which aliquots for Curves I and II were taken. Ordinate, number of crescents induced by 200 units/ml. penicillin for 4.5 minutes (logarithmic scale). One unit equals 5×10^7 crescents/ml.

component formation is taking place should therefore differ in their inducibility depending upon whether cell division is blocked or allowed to proceed.

In a continuous induction experiment, 40 units/ml. penicillin was added to aliquots taken from a synchronized culture at 0, 20, 40, 60, and 80 minutes following the addition of casamino acids to the culture. The progressive induction of crescents was followed in each of the aliquots. The induction curves obtained for the 0, 40, and 80 minute aliquots were identical as were the curves of the 20 and 60 minute aliquots. The behavior of the 0 and 20 minute aliquots are shown in Fig. 3. The results of a short-term induction experiment on the control culture are also given. As may be seen, the temporary reduction in crescents at 36 minutes in Curve C is accompanied by a cessation of the induction of crescents in the 0 minute aliquot (Curve I), the number of crescents in the latter remaining essentially constant for the next 35 minutes, after which the crescent count increases to the expected value. On the other hand, the induction of crescents in the 20 minute aliquot continues unaffected as is to be expected in cells in which the division of cells assures a constant number of penicillin-sensitive components/cell unit.

The Effect of Short-term Doses of Penicillin upon Cells Undergoing Continuous Induction

It is difficult to explain the results of the continuous induction experiments on the basis of a transitory period in the cell's life cycle, during which the cell is refractory to penicillin. Nevertheless, it is possible to conceive of such a refractory period being prolonged by blocking the cell-division process.

To eliminate this possibility, an experiment was carried out in which aliquots from a synchronized culture, undergoing continuous induction in the presence of 40 units/ml. of penicillin, were subjected to short-term inducing doses of 200 units of penicillin for 4.5 minutes (Fig. 4). To reduce the number of crescents induced at the time of cell division, continuous induction was initiated by the addition of penicillin to the culture at 9 minutes after addition of casamino acids, instead of at 0 minutes. (It will be recalled that at this time addition of 40 units/ml. of penicillin still succeeds in blocking the division process.) As may be seen from Curve III, the results obtained are similar to those obtained by adding penicillin at 0 minutes, with the difference that crescent induction is blocked at a lower level in this experiment than in that shown in Fig. 3 (30% instead of 50%). It will be seen in Curve I that a rapid rise in number of short-term inducible cells takes place, reflecting the cumulative effect of the continuing dose of 40 units/ml. of penicillin. This results in a total induction (by the 200 units – 4.5 minute dose + the continuous 40 unit dose) of 2.65×10^8 crescents or 100% of the cells by 28 minutes, 19 minutes following the initiation of the continuing dose of 40 units/ml. of penicillin. The effect of the short-term dose alone is to induce 40% of the cells (first part of Curve I).

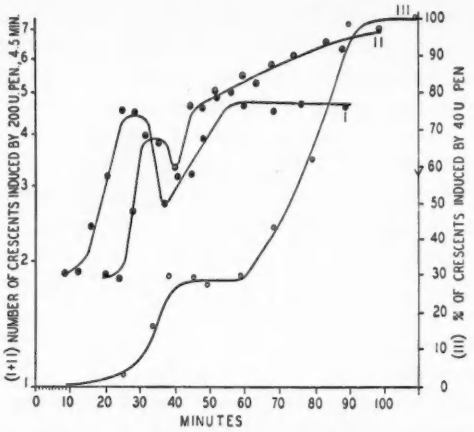

FIG. 4. Crescent inducibility of aliquots taken from a synchronized culture undergoing continuous induction in 40 units/ml. penicillin.

Curve I: Total number of crescents induced by subjecting aliquots taken at intervals from a synchronous culture, undergoing continuous induction by 40 units/ml. penicillin added at *9 minutes* following the onset of synchronized growth, to 200 units/ml. penicillin for 4.5 minutes. Ordinate, total number of crescents formed (logarithmic scale). One unit equals 5×10^7 crescents/ml. Abscissa, time in minutes following the onset of synchronized growth that 200 units/ml. of penicillin was added for 4.5 minutes.

Curve II: Total number of crescents induced by subjecting aliquots taken at intervals from a synchronous culture, undergoing continuous induction by 40 units/ml. penicillin added at *20 minutes* following the onset of synchronous growth, to 200 units/ml. penicillin for 4.5 minutes. Ordinate, as in Curve I. Abscissa, as in Curve I.

Curve III: Continuous induction of crescents by 40 units/ml. penicillin added to the synchronized culture 9 minutes after the onset of synchronous growth. Samples for Curve I were taken from this culture. Ordinate, per cent crescents induced (linear scale) $100\%=2.35\times10^8$ crescents. Abscissa, time in minutes, after onset of synchronous growth, at which penicillin was removed by dilution.

The number of inducible cells (crescent count in Curve I) drops again at 35 minutes simultaneously with the cessation of induction in the continuous induction culture (Curve III). The inducible cell count then rises slowly to reach a value of 100% during the next 15 minutes. The degree to which the number of inducible cells is reduced at 35 minutes is of great interest, being exactly that amount that one would expect if a new component were made at that time, which had not been subjected to the continuous dose of penicillin. Thus, at the onset of the experiment, the short-term inducing dose alone was capable of inducing 9.5×10^7 crescents or about 40% of the cells. At 35 minutes, 30% of the cells had been already induced by the continuous presence of penicillin. If one assumes the original potency to be still in operation for the short-term inducing dose, the probability of a newly synthesized component being effected by penicillin should be 40%. Thus, of the cells which have not yet been induced to form crescents, 40% should now be induced—amounting to 40% of the remaining 70%, or 28%. From this it follows that the total number of cells induced at 35 minutes should be equal to the total number previously induced by 40 units of penicillin alone, 30%, plus the number induced by the short-term penicillin dose, 28%, yielding a

total induction of 58% of the cells in the population. This figure agrees well with the value found experimentally, 1.4×10^8 crescents or 60% of the cells.

Curve II represents the change in inducible cells in the aliquot of the culture to which 40 units/ml. penicillin were added at 20 minutes. (The continuous induction curve for this sample was identical with that of Curve II, Fig. 3, and was therefore not included in this figure.) The results are generally what might be expected in a population in which 60 to 70% of the cells undergo division. This curve is more difficult to evaluate quantitatively, owing to the presence of a mixed population of dividing and non-dividing cells and to a certain amount of variation in the division times of the individual cells. The rapid recovery following the loss of inducibility is to be expected in a population in which most of the cells divide, yielding the original inducible population plus a population of new cells which are not readily inducible. These new cells in turn become inducible, raising the total population of inducible cells.

The results of the experiment shown in Fig. 4 would tend to rule out the possibility of a transitory phase in the cell during which it is refractory to the effects of penicillin. This is particularly clear in a comparison of Curves I and III, which demonstrate that although the continuous induction of the population has been blocked by low concentrations of penicillin, the population remains inducible.

Discussion

The recent work of Weidel and Primosigh (11) has shown that the formation of protoplast-like forms in Gram-negative organisms is due to the inhibition of the synthesis of one of two layers of the cell wall, a layer similar in composition to the cell walls of Gram-positive organisms. Thus, the action of penicillin on Gram-negative organisms would appear to have a similar basis to that shown to exist for certain Gram-positive organisms (9, 8).

In view of the experiments presented, it is difficult to visualize the mechanism of action of penicillin as one whereby penicillin acts as a non-specific inhibitor of "Gram-positive" cell-wall synthesis. To be sure, only the isolation and identification of the component, and demonstration of the ability of the purified component to reverse the biological action of penicillin, would serve to identify conclusively a specific cell component upon which penicillin exerts an effect. Nevertheless, the experiments presented have strongly indicated that the induction of crescent formation *is* the result of the action of penicillin upon some cellular component which carries out a function concerned with the normal bacillary growth of the cell. Penicillin destroys the functioning of this component, thereby disrupting the normal growth process. The presence within the cell of an additional such component upon which penicillin has not acted will allow the cell to continue its normal growth, despite the destruction within the cell of other similar components by penicillin.

Such a hypothesis explains the reduction in the number of crescents induced in synchronized cultures by short-term doses of penicillin during the period immediately prior to cell division (Fig. 1). It also explains in a quantitative as well as qualitative manner the varying inducibility of cultures in which penicillin has blocked the division process (Figs. 3 and 4).

On the basis of the latter data, it appears unlikely that the induction of crescent formation in strain LB is the result of inducing an intracellular enzyme capable of some type of degradative action on the cell wall. The latter idea, suggested in connection with the action of penicillin on *E. coli* (10), would not explain the sudden cessation of crescent induction observed in Figs. 3 and 4.

The nature of the component of LB affected by penicillin is not known, although it would appear that it is synthesized immediately before cell division. This knowledge should greatly aid in experiments on the isolation and identification of the substance involved. The abrupt manner in which this substance is synthesized in the cell-division cycle suggests that it is a highly specific substance. If this substance is protein in nature, it could only represent a small fraction of the cellular protein, since experiments have shown that protein synthesis in *A. fecalis* takes place during a considerable period of time, both before and after cell division (5). Finally, it should be remembered that the site of penicillin action (and probably of the component acted upon) is not necessarily in the cell wall, since cells of *A. fecalis* LB which have lost a large portion of their cell wall can continue to grow in tryptone and will depend for their loss of cell wall on the continued inducing action of penicillin (4).

As noted previously (4), a primary effect of penicillin is on the division process. The data presented confirm this and demonstrate that cell multiplication is stopped prior to the induction of crescents and to the cessation of synthesis of the component that penicillin effects in inducing crescent formation. (From the data in Fig. 4, it would appear that the synthesis of this component is halted after the first cycle of multiplication, in that neither Curves I nor II show any dip at 80 minutes.) Death of the cell appears to be the last effect noted in that no appreciable inactivation of the cell population was noted after 80 minutes in the presence of 40 units/ml. of penicillin (Table I). From the induction curve in Fig. 1, it would appear probable that following penicillin induction no further multiplication of the component on which penicillin acts takes place since, if this were the case, one would not expect the sharp dip immediately prior to cell division which is observed. For the same reason it seems logical to assume that high short-term inducing doses immediately block all further cell division in those cells induced.

It is tempting to speculate on the relation between the effect of penicillin on the cell-division process and its effect in inducing crescent formation. At the present time, it is impossible to say whether or not the same component of the cell is involved in the two processes. Experiments are under way to test this possibility, and it would appear that the use of synchronized cultures will prove a ready tool in finding the answer.

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